Remarks

Amendments

Claims 27 and 56 have been amended to recite "*in vitro*" methods of treating cells by administering a therapeutically effective amount of antisense oligonucleotides. The specification supports the amendment at page 10, lines 22-24: "According to another embodiment of the invention, interference with the expression of MDM2 provides a therapeutic modality. The method can be applied in vivo, in vitro, or ex vivo."

The amendment thus introduces no new matter.

The Rejection of Claims 27, 28, 56, 58, and 60 Under 35 U.S.C. § 112, First Paragraph

Claims 27, 28, 56, 58, and 60 have been rejected under 35 U.S.C. § 112 first paragraph as not adequately described by the specification. Claims 58 and 60 are canceled. Applicants traverse the rejected as applied to amended claims 27, 28, and 56.

Claims 27 and 56 are the independent claims of the rejected claim set. Claim 27 is directed to an *in vitro* method of treating a neoplastic cell. Claim 56 is directed to an *in vitro* method of treating a cell having an amplified human MDM2 gene, elevated expression of human MDM2 mRNA, or elevated expression of human MDM2 protein. Both methods comprise a step of administering to the cell a therapeutically effective amount of antisense oligonucleotides which are complementary to human MDM2 mRNA and which inhibit transcription or translation of a human MDM2 gene.

The Office Action asserts that the claims are not adequately described because the specification fails to provide a sufficient number of representative species of antisense oligonucleotides that will function in the claimed methods. The Office Action asserts:

> The specification discloses no examples of antisense oligonucleotides, thereby failing to set forth any representative species of antisense oligonucleotides from within the broad genus of antisense oligonucleotides as claimed. Moreover, neither the specification nor a search of the prior art at the time the invention as made, provides or points to a specific structure of an antisense oligonucleotide, as claimed, that would correspond with the function as claimed. The specification does not disclose any distinguishing identifying characteristics of the genera of antisense oligonucleotides which can be of any degree of complementarity to any human MDM2 mRNA . . . Additionally, the disclosure of the specification provides no specific guidance as to how one skilled in the art might be reasonably led to a particular species of the invention that would function commensurate with the scope [of] what is now claimed, such that the invention would be complete and ready for patenting.

Office Action at page 6, lines 4-21.

To satisfy the written description requirement of 35 U.S.C. § 112, the specification must describe the claimed invention in sufficient detail that one skilled in the art can reasonably conclude that the inventor had possession of the claimed invention. *Vas-Cath, Inc. v. Mahurkar*, 935 F.2d 1555, 1563-1564 (Fed. Cir. 1991). A specification need not disclose what is well known to those skilled in the art and preferably omits that which is well known to those skilled and already available to the public. *In re Buchner*, 929 F.2d 660, 661 (Fed. Cir. 1991); *Hybritech, Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 1384 (Fed. Cir. 1986).

One of skill in the art, at the time the application was filed, would have understood that the inventors had possession of the recited "antisense oligonucleotides which are complementary to human MDM2 mRNA" for use in the claimed *in vitro* methods of treating cells. The specification discloses the coding sequence of a human *MDM2* gene at SEQ ID NO: 2. One of skill in the art would have understood that applicants had possession of a human MDM2 mRNA sequence based on the disclosure of the coding sequence of a human *MDM2* at SEQ ID NO: 2; a

human MDM2 mRNA sequence is a nucleotide sequence coding for human MDM2. One of skill in the art also would have understood that applicants had possession of sequences complementary to the human MDM2 mRNA sequences because one of skill in the art was apprised of the well-known rules of complementary base pairing, *i.e.*, A is complementary to T and G is complementary to C. The Office Action asserts that the specification does not disclose any distinguishing identifying characteristics of the genera of antisense oligonucleotides which can be of any degree of complementarity. The claims recite that the antisense oligonucleotides are "complementary to human MDM2 mRNA." An antisense oligonucleotide that is complementary to human MDM2 mRNA is one that follows the rules of complementary base pairing for each nucleotide in the antisense oligonucleotide sequence. There are no degrees of complementarity.

Furthermore, one of skill in the art would have understood that applicants had possession of oligonucleotides comprising a nucleotide sequence complementary to the human mRNA sequences and "which [would] inhibit transcription or translation of a human MDM2 gene" because the synthesis of such oligonucleotides was also well-known in the art at the time the application was filed. Exhibits A-L demonstrate that antisense oligonucleotides complementary to a selected mRNA and that inhibited gene transcription or translation were readily made based on the sequence of the gene before the effective filing date of the application, April 7, 1992.

Burch *et al.* (*J. Clin. Invest. 88* (1991):1190-1196; Exhibit A) teach successful synthesis and use of antisense oligonucleotides to block interleukin (IL)-1 receptor expression in cultured cells. Burch *et al.* teach: "We have demonstrated that oligodeoxynucleotides antisense to the IL-1 receptor block the expression of IL-1 receptors and the biological effects of IL-1 on cultured cells." Page 1194, column 1, lines 10-12. Thus, Burch *et al.* teach antisense oligonucleotides

complementary to a selected mRNA, *e.g.*, IL-1 receptor, inhibited transcription or translation of a gene.

Harel-Bellan *et al.* (*J. Exp. Med. 168* (1988):2309-2318; Exhibit B) teach successful synthesis and use of antisense oligonucleotides to inhibit expression of IL-2 and IL-4 genes in mouse helper T cell clones: "We have used synthetic oligonucleotides complementary to the 5' end of mouse IL-2 and IL-4 to specifically block the biosynthesis of IL-2 or IL-4 in two murine helper T cell clones from the Th1 or Th2 subset . . . Northern analysis, using cDNA probes specific for the two lymphokines, showed a decrease in the steady-state level of the relevant lymphokine mRNA." Page 2316, lines 2-13. Thus, Harel-Bellan *et al.* teach the synthesis of antisense oligonucleotides complementary to IL-2 and IL-4 mRNA which inhibited transcription or translation of IL-2 and IL-4 genes.

Hambor *et al.* (*Proc. Natl. Acad. Sci. USA. 168* (1988):1237-1245; Exhibit C) teach successful synthesis and use antisense oligonucleotides to inhibit CD8 gene expression in a human cytotoxic T-cell clone. Hambor *et al.* teach, "Effective (>95%), selective, and reversible anti-sense RNA-mediated gene inhibition of a model T-cell-associated molecule (CD8) was achieved in a cytotoxic human T-cell clone by using an EBV episome-based, RSV 3' LTR-driven expression system." Page 4010, lines 8-12 of the abstract. Thus, Hambor *et al.* teach an antisense oligonucleotide complementary to CD8 mRNA that inhibited transcription or translation of the *CD8* gene.

Simons *et al.* (*Cir. Res. 70* (April 1, 1992):835-843; Exhibit D) teach successful use of antisense oligonucleotides to inhibit nonmuscle myosin heavy chain (NMMHC) and c-myb expression in smooth muscle cells (SMCs) *in vitro*. Simons *et al.* teach, "We have used antisense nonmuscle myosin heavy chain (NMMHC) or c-myb phosphorothiolate

oligonucleotides to inhibit proliferation of SMCs in vitro. The suppression of growth in accompanied by reductions in the concentrations of NMMHC and c-myb mRNAs as well as decreases in the levels of the corresponding proteins." Lines 5-9 of the abstract. Thus, Simons *et al.* teach the synthesis of antisense oligonucleotides complementary to NMMHC and c-myb mRNA which inhibited transcription or translation of NMMHC and c-myb genes.

Watson *et al.* (*Cancer Res. 51* (1991):3996-4000; Exhibit E) teach that antisense oligonucleotides targeted to c-myc inhibited c-myc expression in MCF-7 cells *in vitro*. Watson *et al.* teach: "Estrogen induces a 5-fold increase in c-myc protein expression within 90 min in steroid-deprived cells, as detected by Western blot. Prior exposure of MCF-7 cells in 10 microM c-myc antisense oligonucleotide results in up to 95% inhibition of the c-myc protein expression induced by estrogen." Lines 7-10 of the abstract. Thus, Watson *et al.* teach that an antisense oligonucleotide complementary to c-myc mRNA inhibited transcription or translation of the *c-myc* gene.

Sankar *et al.* (*Eur. J. Biochem. 184* (1989):39-45; Exhibit F) teach several antisense oligonucleotides that inhibited encephalomyocarditis virus RNA translation *in vitro*. Sankar *et al.* teach, "We report the inhibition of encephalomycarditis virus (EMCV) RNA translation in cell-free rabbit reticulocyte lysates by antisense oligonucleotides (13-17-base oligomers) complementary to (a) the viral 5' non-translated region, (b) the AUG start codon and (c) the coding sequence." Lines 1-4 of the abstract. Thus, Sankar *et al.* teach three antisense oligonucleotides complementary to encephalomyocarditis virus RNA that inhibited transcription or translation of the encephalomyocarditis virus RNA.

Harel-Bellan *et al.* (*J. Immunol. 140* (1988):2431-2435; Exhibit G) teach that an antisense oligonucleotide targeted to c-myc inhibits expression of c-myc protein in cell lines *in*

vitro. Harel-Bellan *et al.* teach, "The oligonucleotide specificity blocked the de novo synthesis of c-myc protein, induced by PHA in human resting peripheral T cells, without impairing the overall synthesis of other proteins." Lines 4-7 of the Abstract. Thus, Harel-Bellan *et al.* teaches inhibition of transcription or translation of a *c-myc* using an antisense oligonucleotide complementary to *c-myc's* second exon.

Goodchild *et al.* (U.S. Patent No. 4,806,463 filed May 23, 1986; Exhibit H) teach that antisense oligonucleotides targeted to HTLV-III nucleotide sequences inhibit HTLV-III gene expression and virus replication. Goodchild *et al.* teach, "Using the oligodeoxynucleotide sequences described above and in Example 3, it was possible to inhibit HTLV-III replication and gene expression in HTLV-III-infected cells in tissue culture." Column 6, lines 15-18, see also Example 3 and Table 1. Thus, Goodchild *et al.* teach successful synthesis of antisense oligonucleotides complementary to an HTLV-III nucleotide sequence that inhibit transcription or translation of genes.

Morrison (*J. Biol. Chem. 266* (1991):728-734; Exhibit I) teaches that antisense oligonucleotides targeted to basic fibroblast growth factor (bFGF) mRNA inhibit bFGF expression *in vitro*. Morrison teaches that "antisense primer AS-1 (35 μM) significantly reduced bFGF expression in SNB-19 cells. The 67% reduction in bFGF content was paralleled by a 55% reduction in cell number (data not shown), implying that inhibition of SNB-19 cell growth was directly related to the loss of bFGF." Page 731, column 1, lines 16-20. Thus, Morrison also teaches yet another antisense oligonucleotide complementary to an mRNA sequence inhibited gene transcription or translation.

Sumikawa *et al.* (*Proc. Natl. Acad. Sci. USA 65* (1991):213-219; Exhibit J) teach the successful use of each of four antisense oligonucleotides to inhibit transcription or translation of the

acetylcholine receptor coding sequence. Sumikawa et al. teach: "Four antisense RNAs, synthesized from cDNA clones coding for the four subunits of the acetylcholine receptor of *Torpedo* electroplaques, were used to study their effect of the expression of functional *Torpedo* acetylcholine receptors in Xenopus oocytes. All antisense RNAs inhibited the appearance of functional receptors in the oocyte's surface membrane for at least 1 week." Page 1302, lines 1-7 of the abstract. Sumikawa *et al.* teach four antisense oligonucleotides that were successfully synthesized to inhibit transcription or translation of acetylcholine receptor mRNA.

Draper (U.S. Patent No. 5,004,810 filed September 30, 1988; Exhibit K) teaches that antisense oligonucleotides inhibit herpes simplex virus (HSV) replication in cell culture by inhibiting viral gene expression. Draper teaches, "When LTK-cells were pretreated with oligo 293, infected with HSV, then incubated in the presence of oligodeoxyribonucleotide, yields of infectious virus were reduced 50-82% as compared to control levels produced in untreated (no oligo) cells (Table 2)." Column 13, lines 10-15. Draper further teaches that this inhibition of viral replication is due to an inhibition of viral gene expression. Draper teaches, "Thus it would seem that complementary oligodeoxyribonucleotides can be targeted to specifically hybridize to the initiation region for Vmw65 translation and to inhibit viral gene expression." Column 14, lines 35-49. Thus, Draper also teaches that antisense oligonucleotides complementary to Vmw65 mRNA inhibited viral gene transcription or translation.

Flood *et al.* (J. Exp. Med. *172* (1990):115-120; Exhibit L) teaches that antisense oligonucleotides targeted to *Ly-6A* mRNA inhibit expression of the Ly-6A protein in cells *in vitro*. Flood et al. teaches "Antisense oligonucleotides complementary to the 5' end of the mRNA encoding the Ly-6A protein were used to block the expression of the protein. Using this approach we could inhibit the expression of Ly-6A by 60-80% in antigen-primed lymph node (LN) T cells as

well as in the D10 T cell clone." Page 115, lines 1-4 of the abstract. Thus, Flood *et al.* teach a further antisense oligonucleotide complementary to a selected mRNA, which inhibits transcription of translation of the gene.

As can be seen by Exhibits A-L, it was known in the art at the time the application was filed that antisense oligonucleotides capable of inhibiting transcription or translation of a gene could be successfully synthesized and used. Thus, the application disclosure of a coding sequence of human MDM2 at SEQ ID NO: 2 would have conveyed to one of skill in the art that applicants had possession of "antisense oligonucleotides which are complementary to human MDM2 mRNA and which inhibit transcription or translation of a human MDM2 gene" in the claimed *in vitro* methods of treating cells. The claims are therefore adequately described.

Applicants respectfully request withdrawal of this rejection.

The Rejection of Claims 27, 28, 56, 58, and 60 Under 35 U.S.C. § 112, First Paragraph

Claims 27, 28, 56, 58, and 60 have been rejected under 35 U.S.C. § 112 first paragraph as not enabled. Claims 58 and 60 are canceled. Applicants traverse the rejection as it is applied to amended claims 27, 28, and 56.

To satisfy the enablement requirement, the specification of a patent must teach those skilled in the art how to make and use the full scope of the claimed invention without undue experimentation. *In re Wright*, 999 F.2d 1557, 1561 (Fed. Cir. 1993). That some experimentation may be required is not fatal; the issue is whether the amount of experimentation required is "undue." *In re Vaeck*, 947 F.2d 488, 495 (Fed. Cir. 1991). The test is not merely quantitative, because a considerable amount of experimentation is permissible if the experimentation is merely routine or if the specification in question provides a reasonable

amount of guidance with respect to the direction in which the experimentation should proceed. *In re Wands*, 858 F.2d 731, 737 (Fed. Cir. 1988).

The Office Action asserts that the claims are not enabled because the specification does not provide examples of antisense oligonucleotides that inhibit transcription or translation of human MDM2 mRNA or guidance as to how to identify these antisense oligonucleotides. The Office Action asserts:

> The specification as filed provides no support for claims to methods of treatment comprising administering antisense oligonucleotides of the invention that inhibit the transcription of translation of human MDM2 mRNA. The specification as filed provides no examples of treatment comprising administering antisense oligonucleotides of the invention and no guidance as to how to make or use the antisense oligonucleotides of the invention that will function to provide a treatment as claimed . . . Moreover, the specification as filed provides no specific guidance that would allow the skilled artisan to recognize antisense oligonucleotides that will function in the methods of treatment as claimed.

Office Action at page 10, line 17 to page 11, line 10. The Patent Office further cites references Agrawal, Rojanasakul, Opalinska, Jen, and Check as evidence that the claims are not enabled. The Office Action asserts:

> At the time the instant invention set forth in claims 27, 28 and 56 was made and even 6 years later, at the time the invention set forth in claims 56 and 58 was made, such obstacles [problems with delivery (including uptake by cells) and target accessibility] were still relevant to the enablement of antisense inhibition of gene expression *in vitro* (see below: Agrawal et al., Rojanaskul, Opalinka et al., Jen et al., and Check).

Office Action at page 11, lines 18-23.

The Agrawal, Rojanaskul, Opalinska, Jen, and Check references teach obstacles to using antisense oligonucleotides *in vitro*. However, the Agrawal, Rojanaskul, Opalinska, Jen, and Check references also teach that despite these obstacles, antisense oligonucleotides

complementary to a nucleotide sequence successfully inhibited a gene's transcription or translation *in vitro*.

- Opalinska teaches:

  > Another problem in this field is the limited ability to deliver nucleic acids into cells and have them reach their target . . . After internalization, confocal and electron microscopy studies have indicated that the bulk of the oligonucleotides enter the endosome-lysosome compartment, in which most of the material becomes either trapped or degraded . . . Nevertheless, oligonucleotides can escape from the vesicles intact, enter the cytoplasm and then diffuse into the nucleus, where they presumably acquire their mRNA, or in the case of decoys, protein target.

  Page 511, column 2, lines 13-29. Opalinska also teaches, "Nucleic-acid-mediated gene silencing has been used with great success in the laboratory." Page 511, column 1, lines 11-12.

- Rojanaskul teaches, at Table 1, of twenty-seven references which teach inhibition of a gene's transcription or translation with antisense oligonucleotides.

- Check teaches, "RNAi may work like a charm in Petri dishes—but what about in live animals?" Page 11, column 1, lines 6-7.

- Agrawal teaches, "There are numerous examples in which PS-oligonucleotides of varying lengths and base compositions have been employed to inhibit the translation of cellular or foreign gene by an antisense mechanism." Page 73, column 1, lines 8-11.

Thus, Agrawal, Rojanaskul, Opalinska, Jen, and Check teach that antisense oligonucleotides complementary to a nucleotide sequence are able to inhibit a gene's transcription or translation despite any potential obstacles to the use of the antisense oligonucleotides *in vitro*.

Furthermore, and contrary to the assertion in the Office Action, one of skill in the art

would have been able to make and use the recited "antisense oligonucleotides which are complementary to human MDM2 mRNA and which inhibit transcription or translation of a human MDM2 gene" in the claimed methods without resorting to undue experimentation. It was well known in the art, at the time the application was filed, how to make and use antisense oligonucleotides complementary to an mRNA sequence which inhibited transcription or translation of the mRNA's corresponding gene. Before the effective filing date of the application, April 7, 1992, the art taught how to make and use antisense oligonucleotides that inhibited transcription or translation of a target gene based on the target gene's sequence. See Exhibits A-L, discussed briefly below:

- Burch *et al.* (Exhibit A) teach that antisense oligonucleotides to the IL-1 receptor gene block its expression in cultured cells *in vitro*.
- Harel-Bellan *et al.* (Exhibit B) teach that antisense oligonucleotides targeted to IL-2 and IL-4 genes inhibit IL-2 and IL-4 gene expression in mouse helper T cell clones.
- Hambor *et al.* (Exhibit C) teach that antisense oligonucleotides targeted the CD8 gene inhibit CD8 gene expression in a human cytotoxic T-cell clone.
- Simons *et al.* (Exhibit D) teach that antisense oligonucleotides targeted to nonmuscle myosin heavy chain (NMMHC) and c-myb genes inhibit NMMHC and c-myb expression in smooth muscle cells (SMCs) *in vitro*.
- Watson *et al.* (Exhibit E) teach that antisense oligonucleotides targeted to c-myc inhibited c-myc expression in MCF-7 cells *in vitro*.
- Sankar *et al.* (Exhibit F) teach that antisense oligonucleotides designed to target encephalomycarditis virus genes inhibited encephalomyocarditis virus RNA translation *in vitro*.
- Harel-Bellan *et al.* (Exhibit G) teach that an antisense oligonucleotide targeted to c-myc inhibits expression of c-myc protein in human resting peripheral T cells *in vitro*.
- Goodchild *et al.* (Exhibit H) teach that antisense oligonucleotides targeted to HTLV-III nucleotide sequences inhibit HTLV-III gene expression and virus replication *in vitro*.
- Morrison (Exhibit I) teaches that antisense oligonucleotides targeted to bFGF inhibit bFGF expression in a SNB-19 cell line *in vitro*.
- Sumikawa *et al.* (Exhibit J) teach four antisense oligonucleotides targeted to an acetylcholine receptor coding sequence that inhibited transcription or translation of the

acetylcholine receptor coding sequence in *Xenopus* oocytes *in vitro*.

- Draper (Exhibit K) teaches that antisense oligonucleotides targeted to HSV inhibit HSV gene expression and replication in an LTK cell line *in vitro*.
- Flood *et al.* (Exhibit L) teaches that antisense oligonucleotides targeted to the *Ly-6A* gene inhibit expression of the Ly-6A protein in LN and D10 cells *in vitro*.

Exhibits A-L demonstrate that at the time of the invention the skilled artisan knew how to make and use antisense oligonucleotides that inhibited a gene's transcription or translation. It therefore would not have required undue experimentation for one of skill in the art to make and use "antisense oligonucleotides which are complementary to human MDM2 mRNA and which inhibit transcription or translation of a human MDM2 gene" for use in the claimed methods. Furthermore, any experimentation that would have been required to identify the recited antisense oligonucleotides would merely have been routine. As demonstrated in Exhibits A-L, the art routinely engaged in experimentation using antisense oligonucleotide that inhibited transcription or translation of a gene. The claimed methods are therefore enabled.

Applicants respectfully request withdrawal of this rejection.

The Rejection of Claims 58 and 60 Under 35 U.S.C. § 102(e)

Claims 58 and 60 are rejected under 35 U.S.C. § 102(e) as being anticipated by Miraglia *et al.* (U.S. Patent Number 6,184,212). Claims 58 and 60 are canceled by this amendment. Thus, the rejection is moot.

Applicants respectfully request withdrawal of this rejection.

Respectfully submitted,

Dated: May 31, 2005 By: _____
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Oligonucleotides Antisense to the Interleukin 1 Receptor mRNA Block the Effects of Interleukin 1 in Cultured Murine and Human Fibroblasts and in Mice

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Abstract

Phosphodiester and phosphorothioate oligodeoxynucleotides (18 mers) were constructed antisense to sequences of the recently cloned murine and human IL-1 receptors. Murine antisense oligonucleotides inhibited IL-1–stimulated PGE_2 synthesis by murine fibroblasts in culture in a time (days) and concentration-dependent (3 μM–30 μM) fashion. Murine sense oligonucleotide and an oligonucleotide antisense to human IL-1 receptor were without effect. Moreover, murine antisense oligonucleotides did not affect tumor necrosis factor- or bradykinin-stimulated PGE_2 synthesis by murine fibroblasts. Similarly, antisense oligonucleotides to the human, but not the murine, IL-1 receptor inhibited IL-1–stimulated PGE_2 synthesis by cultured human fibroblasts. The attenuation of the cellular response to IL-1 caused by the antisense oligonucleotides correlated with a loss in cell surface receptors for IL-1, without any change in the number of bradykinin receptors on these cells. When antisense oligonucleotides were encapsulated in liposomes, they blocked completely the appearance of newly synthesized IL-1 receptors and IL-1–stimulated PGE_2 synthesis. In mice, subcutaneous injection with an oligonucleotide antisense to the murine IL-1 receptor markedly inhibited the infiltration of neutrophils in response to subsequent injection of IL-1. These data suggest that antisense oligodeoxynucleotides may share a role in the design of antiinflammatory therapeutics. (*J. Clin. Invest.* 1991. 88:1190–1196.) Key words: inflammation • dermatitis • neutrophil infiltration

Introduction

IL-1, a cytokine released from a variety of cells in response to inflammatory insult, is implicated in the pathogenesis of several chronic inflammatory diseases, including rheumatoid arthritis (1). IL-1 elicits its effects by binding to specific cell-surface receptors on a broad spectrum of cell types. IL-1 receptors expressed by T lymphocytes, endothelial cells, and fibroblast-like cells (so called type I receptors) are thought to be identical, whereas IL-1 receptors on macrophages and B lymphocytes (type II receptors) are different (2–4). Recently, type I IL-1 receptors have been cloned from human and murine T lymphocyte and human fibroblasts (5–7) and the amino acid sequences were found to be highly conserved between species.

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We wish to study the expression and activation of IL-1 receptors in cells and to assess the role of IL-1 in physiological and pathological processes. Evidence that IL-1 is implicated in the pathogenesis of certain inflammatory diseases has generated great interest in developing therapeutic agents which inhibit the biological effects of this cytokine. Antisense oligodeoxynucleotides present a potential alternative therapeutic approach. Antisense oligodeoxynucleotides have been used to block translation of proteins both in cell-free translation systems and in cultured cells (8). Use of natural phosphodiester-containing oligonucleotides (O^1-oligos) in studies using intact cells or animals is limited by their rapid metabolism by endogenous nucleases (9). However, phosphorothioate oligonucleotides (S-oligos) are more nuclease-resistant (10), making them potentially useful in vivo, even though they are taken up into cells more slowly (9). To date, however, most studies using antisense oligodeoxynucleotides have focused on the inhibition of expression of viral genes and oncogenes. To assess the capability that these agents may possess to modulate cytokine-mediated responses, we have designed species-specific oligodeoxynucleotides antisense to mRNA sequences for the human and murine IL-1 receptors. We have used these agents to study aspects of cellular and species-specific targeting and their ability to attenuate IL-1 mediated responses in vitro. In addition, we have extended these findings to an in vivo model of cytokine-mediated inflammation to block neutrophil infiltration in response to local administration of IL-1 in mouse dermal tissue.

Methods

Cell culture. 3T3 fibroblasts were cultured as described in DMEM supplemented with 10% calf serum (11) to confluency in 96-well plates. Human dermal fibroblasts were obtained from Clonetics Corp., San Diego, CA, and were cultured in DMEM containing 10% fetal bovine serum.

IL-1 binding. IL-1 binding experiments were performed as described (12). Confluent cultures in 24-well plates were washed twice with HBSS containing 20 mM Hepes, pH 7. To each dish was added 200 μl ice-cold HBSS containing 20 mM Hepes, 1 mg/ml BSA, and 50 nCi human recombinant [^{125}I]IL-1α (New England Nuclear, Boston, MA). For Scatchard analyses, unlabeled IL-1α (Boehringer Mannheim Corp., Indianapolis, IN) was added to vary concentration. Nonspecific binding was determined in the presence of 2 nM IL-1β (Boehringer Mannheim). To terminate experiments, the cultures were incubated on ice for 60 min, then each culture was washed four times with 10 ml ice-cold HBSS containing 1 mg/ml BSA. Cells were removed from plates using HBSS containing trypsin. Cell-associated radioactivity was determined using a gamma counter and was ~ 3,000 dpm/well in the absence of added unlabeled IL-1 and 100–150 dpm in the presence of 2

1. *Abbreviations used in this paper:* h, human; m, murine; O, phosphodiester; S, phosphorothioate.

nM unlabeled IL-1β. K_d and maximal binding capacity (B_{max}) were determined using LIGAND (Biosoft, Milltown, NJ) (13).

Bradykinin binding. Bradykinin receptor binding to cell membranes was carried out as described (11). Cells were scraped into 100 vol of a buffer composed of 25 mM N-tris(hydroxymethyl)methyl-2-aminoethane-sulfonic acid, 0.2 g/liter 1,10-phenanthroline, pH 6.8, and homogenized using a Polytron Tissumizer (Brinkmann Instruments, Inc., Westbury, NY) at setting 6 for 15 s. The homogenate was centrifuged at 50,000 *g* for 10 min, the supernatant discarded, and the pellet resuspended into the same buffer also containing 1 g/liter BSA and 0.14 g/liter bacitracin. To assay tubes were added [^{3}H]bradykinin, the amount of radioactivity being varied in the tubes to vary concentration for Scatchard analysis. Nonspecific binding was determined in the presence of 10 μM NPC 567, a bradykinin antagonist (11). Assays were terminated by filtration through glass fiber filters that had been soaked in 2 g/liter polyethyleneimine for 1 h, and washed four times with 4 ml of ice-cold 50 mM Tris, pH 7.4.

PGE_2 synthesis. PGE_2 was quantitated from direct aliquots of media using a radioimmunoassay as previously described for these cells and media (11). Reagents were from Advanced Magnetics, Inc., Cambridge, MA, and control experiments demonstrated that none of the experimental agents (IL-1, tumor necrosis factor, bradykinin, or antisense oligonucleotides) affected the assay.

Liposomes. Liposomes were constructed of phosphatidylserine (14), loaded with oligonucleotides, and fused to the cells (9). Phosphatidylserine (Avanti Polar Lipids, Inc., Birmingham, AL), 5 mg in 1 ml chloroform, was placed into a spherical flask and, under a nitrogen atmosphere, the chloroform was removed using a rotary evaporator, leaving a film of lipid. The lipid was resuspended in 0.2 ml EDTA buffer (100 mM NaCl, 2 mM histidine, 2 mM Tris acid, 0.1 mM EDTA, pH 7.4) with vigorous vortexing to form multilamellar vesicles. Then $CaCl_2$ was added to 10 mM to form cochleate bodies. Oligonucleotide was added and the mixtures were allowed to stand at room temperature for 1 h. EDTA was then added to 15 mM and the pH adjusted to 7 to cause liposome formation. This solution was allowed to stand for 30 min. Liposomes were concentrated by centrifugation at 100,000 *g* for 30 min at room temperature and gently washed with HBSS. For lipofusion, cells were rinsed with HBSS, then incubated in HBSS containing 2 mM calcium and 0.1 mM magnesium for 30 min. Liposomes were added to a concentration of 1.25 mg/ml lipid and incubated for 30 min. To the cultures was then added a volume of polyethylene glycol (6,000 average molecular mass) equal to the salt solution in the well (resulting in 50% polethylene glycol) for 1 min, followed by addition of culture medium containing serum, and careful washing of the wells three times to remove the polyethylene glycol. In an experiment to determine the efficiency of oligonucleotide incorporation, oligonucleotide was ^{32}P-endlabeled before incorporation into liposomes. After one wash 12% of radioactivity was retained in the liposome pellets. Repeated washing demonstrated that 6–7% of radioactivity was stably retained.

Labeling neutrophils. In the experiments in mice, in vivo, the day before IL-1 was to be administered, animals received 10 μCi [^{3}H]thymidine i.p. to label circulating neutrophils. Using this method, 24 h after injection, virtually all radioactivity in the blood is contained within the DNA of neutrophils (15). Hair was shaved from the backs before the beginning of the experiment. IL-1 was administered intradermally 1 cm lateral to the spine and, 4 h later, animals were killed by cervical dislocation. Skin was removed and 8 mm punch biopsies were obtained. These were dissolved in Protosol (New England Nuclear) overnight, then radioactivity was determined by liquid scintillation counting. Background radioactivity was determined by taking biopsies from areas that had been injected intradermally with saline not containing IL-1. Each animal served as its own control in that murine antisense oligonucleotides were injected on one side of the midline whereas on the other side, injections contained the human antisense oligonucleotide, which is inactive in mouse cells in culture (see *Results*).

Oligonucleotides. Oligodeoxynucleotides were synthesized (Synthecell Corp., Rockville, MD) complementary to unique species-specific coding regions of mRNA for murine and human IL-1 receptors. These included 18-base regions at the initiation codon (mO-oligo-1,5′-CACTTTCATATTCTCCAT, complementary to bases −57 to −40 of the murine sequence [6], and mS-oligo-1, the analogous phosphorothioate analog; hO-oligo-1,5′-TCTGAGTAACACTTTCAT, complementary to bases −51 to −34 of the human IL-1 receptor sequence [5], and hS-oligo-1, the analogous phosphorothioate analogue), an internal open reading frame site (mS-oligo-2, a phosphorothioate analogue of sequence 5′-GAGACAAATGAGCCCCAG, complementary to bases −36 to −19 of the murine sequence), and a region immediately 5′ to the termination codon (mS-oligo-3, a phosphorothioate analogue of sequence 5′-GCCGAGTGGTAAGTGTGT complementary to bases 1654–1671 of the murine sequence). Two sense phosphorothioate oligonucleotides were used, equivalent to mS-oligo-1,5′-ATGGAGAATATGAAAGTG, and hS-oligo-1,5′-ATGAAAGTGTTACTCAGA as controls.

Results

Time course for inhibition of IL-1–stimulated PGE_2 synthesis by antisense oligonucleotides. In initial experiments, both mO-oligo-1 and mS-oligo-1, 10 μM, inhibited IL-1–stimulated PGE_2 synthesis in cultures of murine fibroblasts grown in media with serum that had been heat-inactivated to denature nucleases (16) (Fig. 1 *A*). The O-oligo was more acute in effect,

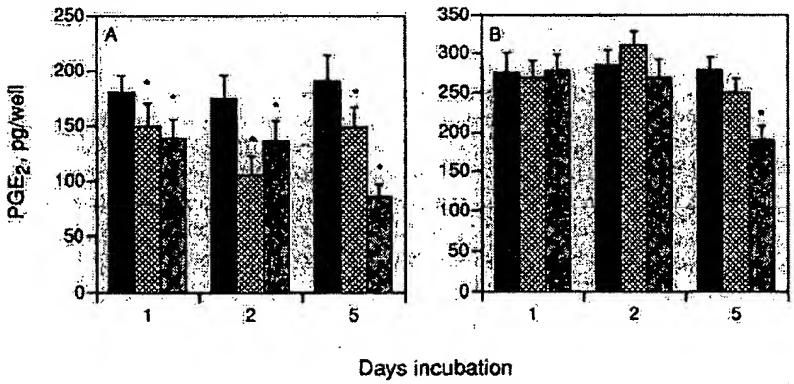

Figure 1. Time course for inhibition of IL-1–stimulated PGE_2 synthesis by antisense oligonucleotides in murine fibroblasts. 3T3 fibroblasts were cultured to confluency, then 10 μM mO-oligo-1 or mS-oligo-1 was added. After incubation for the indicated times, the media were replaced and IL-1α, 100 U/ml was added, and cultures were incubated for 4 h. Then media were collected and assayed for PGE_2. IL-1 stimulation in control cultures (*filled bars*), and in cultures incubated with mO-oligo-1 (*crossed bars*) or mS-oligo-1 (*stippled bars*). Basal synthesis was 42–56 pg/well. *$P < 0.05$ compared to no oligonucleotide by Student's *t* test for paired data. (*A*) Cultures contained 10% heat-inactivated calf serum. (*B*) Cultures contained 10% calf serum that had not been heat inactivated. Data are mean±SEM for triplicate cultures.

while the effect of the S-oligo became more pronounced over a longer period of time. In culture media containing serum that had not been heat-inactivated the O-oligo was without effect, while the S-oligo was still capable of some inhibition of the biological effect of IL-1 (Fig. 1 *B*).

Concentration response for inhibition of IL-1–stimulated PGE_2 synthesis by antisense oligonucleotides. To compare the relative potencies of several antisense oligonucleotides directed to different regions of mRNA for IL-1 receptors, murine fibroblasts were incubated with various concentrations of S-oligos for 48 h. mS-oligo-1, a sequence antisense to the initiation site, was more potent at inhibiting IL-1–stimulated PGE_2 synthesis than mS-oligo-2, which is antisense to a sequence immediately downstream from mS-oligo-1 (Fig. 2 *A*). At a concentration as low as 3 μM, mS-oligo-1 significantly inhibited IL-1–stimulated PGE_2 synthesis. mS-oligo-3, which is antisense to a sequence near the termination codon of the IL-1 receptor mRNA, was without effect on IL-1–stimulated PGE_2 synthesis.

Inhibition of IL-1 receptor-mediated PGE_2 synthesis by the antisense oligonucleotides was selective. While mS-oligo-1 blocked IL-1–stimulated PGE_2 synthesis, it was without effect on tumor necrosis factor- or bradykinin-induced PGE_2 synthesis. Tumor necrosis factor (Genzyme Corp., Cambridge, MA), 10 nM, stimulated PGE_2 synthesis from 45±6 to 216±15 pg/well in 4 h in control cultures, and from 38±10 to 210±18 pg/well in cultures that had been incubated with 30 μM mS-oligo-1 for 2 d. Bradykinin, 1 μM, stimulated PGE_2 synthesis from 12±3 to 44±6 pg/well in 5 min, and from 15±5 to 47±5 pg/well in cultures incubated with 30 μM mS-oligo-1 for 2 d. In addition, neither hS-oligo-1, an antisense oligonucleotide directed to the initiation codon region of the human IL-1 receptor, nor the sense oligonucleotide complementary to mS-oligo-1, had any effect on IL-1–stimulated PGE_2 synthesis in murine fibroblasts (Fig. 2 and data not shown). Analogous to murine fibroblasts, the response of human dermal fibroblasts to IL-1 was inhibited by incubation with hS-oligo-1, but not by mS-oligo-1 or the sense oligonucleotide complementary to hS-oligo-1 (Fig. 2 *B* and data not shown).

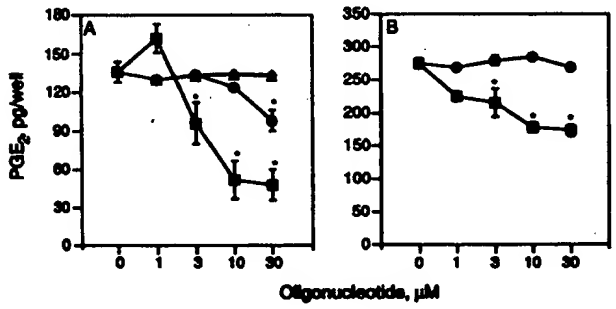

Figure 2. Concentration response for inhibition of IL-1–stimulated PGE_2 synthesis by oligodeoxynucleotides in murine and human dermal fibroblasts. Confluent cultures were incubated with the indicated S-oligos for 48 h, then media were replaced and IL-1, 100 U/ml, was added and cultures were incubated for 4 h before collection of media for assay of PGE_2. (*A*) IL-1–stimulated PGE_2 synthesis in 3T3 fibroblasts in the presence of mS-oligo-1 (■), mS-oligo-2 (●), mS-oligo-3 (▲), or hS-oligo-1 (♦). Data are mean±SEM for triplicate cultures. (*B*) IL-1–stimulated PGE_2 synthesis in human dermal fibroblasts in the presence of hS-oligo-1 (■) or mS-oligo-1 (●). Data are mean±SEM for triplicate cultures. $^*P < 0.05$ compared to no oligonucleotide.

Antisense oligonucleotides reduce expression of IL-1 surface receptors. The attenuation of the cellular response to IL-1 caused by the antisense oligonucleotides correlated with a loss in cell surface receptors for IL-1 in murine fibroblasts. The number and affinity of IL-1 receptors were assessed in murine fibroblasts treated with mS-oligo-1 for 48 h and compared to control fibroblasts. Scatchard analysis demonstrated that the affinity of IL-1 receptors was not altered by treatment with mS-oligo-1, but receptor number was decreased to only 55% of control (Fig. 3). In contrast, mS-oligo-1 did not affect the number of receptors for bradykinin (Fig. 4), demonstrating that the effects of the antisense oligonucleotides were specific to the IL-1 receptor and not due to a cytotoxic effect on the cells.

Encapsulation in liposomes enhances the activity of S-oligos. The time course over which antisense oligonucleotides inhibited cellular responsiveness to IL-1 may have reflected slow turnover of receptors at the cell surface or slow uptake of the oligonucleotides, especially the S-oligos, into cells. To gain insight into which mechanism was predominant, we enhanced delivery of hS-oligo-1 by liposome encapsulation and fusion to human dermal fibroblasts under conditions of steady-state (basal) turnover of receptors and in cells undergoing rapid turnover of their IL-1 receptors. Thus, in the latter case, we treated human fibroblasts for 18 h with 1 nM IL-1 to downregulate IL-1 receptors and IL-1 was then removed to allow recovery of receptors via new receptor synthesis (17–19). We included hS-oligo-1 free in solution or encapsulated in liposomes, to determine whether liposomal delivery of the oligonucleotide into cells could block recovery of responsiveness to IL-1. In cells that had not been exposed to IL-1 to initiate receptor turnover, these short-term incubations of cultures with either free hS-oligo-1 or mS-oligo-1 did not affect response to IL-1 as would be predicted from the data in Fig. 1. Of interest, exposure of cells during steady-state turnover of IL-1 receptors to liposome-encapsulated hS-oligo-1 was also without short-term effect on response to IL-1 (Table I). If, however, cells were exposed to liposome-encapsulated hS-oligo-1 during a period of new receptor expression, complete blockade of the ability of cells to recover responsiveness to activation of IL-1 receptors was observed. No effect of hS-oligo-1 free in solution was seen under these conditions.

Parallel with the studies of recovery of IL-1 responsiveness in the downregulated fibroblasts, we performed IL-1 receptor binding experiments to assure that receptor number correlated with biological responsiveness. Receptor binding studies were carried out as in Fig. 3, but monitoring only specific binding as described (18). Thus, radiolabeled IL-1 (10 nCi) was added to each well in the absence or presence of 2 nM unlabeled IL-1 to account for nonspecific binding. Total specific binding was defined as binding in the presence of radiolabeled IL-1 alone minus binding in the presence of 2 nM unlabeled IL-1. Similar to the biological response, incubation of cells with IL-1 for 18 h was associated with a decrease in receptor number which largely recovered 4 h after removing IL-1 (Table II). Adding 30 μM hS-oligo-1 free in solution to the cultures during the recovery period did not prevent recovery of receptor number, however, enhancing the delivery of the oligonucleotide into the cells using liposomes completely blocked recovery of IL-1 receptors: in cells to which empty liposomes had been fused at the beginning of recovery time, specific binding returned to control levels, while in cells that had been fused with liposomes containing a final concentration of 30 μM hS-oligo-1, specific

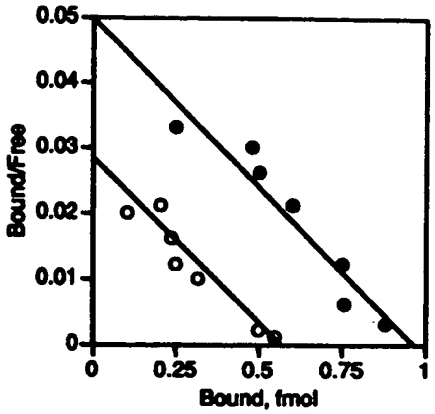

Figure 3. Scatchard analysis of binding of human recombinant IL-1α to cell surface receptors on 3T3 fibroblasts. Illustrated are results from a single experiment. K_d and B_{max} (from three separate experiments) were 145±26 pM and 1.01±0.09 fmol (2,678 receptors/cell) in control cultures (•) and 132±30 pM and 0.56±0.14 fmol in cultures incubated with mS-oligo-1 (30 μM for 2 d) (○). In cultures incubated with hS-oligo-1, 30 μM, for 2 d, K_d was 165±20 pM and B_{max} was 1.06±0.19 fmol.

binding was unchanged from downregulated levels. Thus the half-life of IL-1 receptors at the cell surface play a major role in the biological efficacy of antisense oligonucleotides although attenuation of expression of receptors is considerably augmented using liposome delivery under conditions of new receptor synthesis.

Antisense oligonucleotides block IL-1 effects in mice. Additional experiments were performed to determine whether the effects of the antisense oligonucleotides on IL-1 receptor function in cultured cells could be extended in vivo to an animal model of cytokine-mediated inflammatory response. Subcutaneous injection of IL-1 in mice causes neutrophil infiltration at the site of injection (20, 21). Mice were injected subcutaneously with either mS-oligo-1 or hS-oligo-1 at 24-h intervals over a period of 3 d. 24 h before subsequent subcutaneous

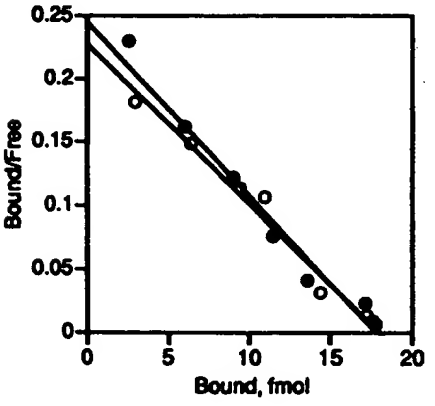

Figure 4. Scatchard analysis of binding of bradykinin to membranes from 3T3 fibroblasts. Illustrated are the results from a single experiment. In membranes from control cultures (•) (n = 3) there were 2,960±410 receptors per cell with K_d of 112±16 pM compared to 3,045±360 receptors per cell with K_d of 125±20 pM in membranes from cells incubated with 30 μM mS-oligo-1 (○) for 2 d.

Table I. Liposome Delivery of S-oligos to Human Dermal Fibroblasts IL-1-induced PGE_2 Synthesis

Condition	PGE_2
	pg/well
Basal receptor turnover	
Basal	46±10
IL-1 18 h, wash 8 h	52±12
no IL-1, wash with media 8 h, IL-1 4 h	744±22
no IL-1, incubate 8 h with liposome hS-oligo-1, IL-1 4 h	695±48
no IL-1, incubate 8 h with liposome mS-oligo-1, IL-1 4 h	718±42
Receptor downregulation/accelerated receptor reappearance	
IL-1 18 h, wash 8 h, IL-1 4 h	398±25
IL-1 18 h, wash 8 h with hS-oligo-1, IL-1 4 h	385±32
IL-1 18 h, wash 8 h with mS-oligo-1, IL-1 4 h	376±36
IL-1 18 h, wash 8 h with liposome hS-oligo-1	68±15
IL-1 18 h, wash 8 h with liposome mS-oligo-1, IL-1 4 h	285±40
IL-1 18 h, wash 8 h with liposome hS-oligo-1, IL-1 4 h	74±25

Data are mean±SEM of triplicate wells for each condition. Human dermal fibroblasts grown to confluency in serum-free media (Clonetics Corp.) in 96-well plates were exposed to human recombinant IL-1 (Boehringer Mannheim), 100 U/ml, for 18 h. The cultures were then washed four times with media to remove IL-1 and incubated in IL-1–free media for 8 h. The media contained oligonucleotides, 30 μM, or liposomes loaded with oligonucleotides, 30 μM final concentration in the culture wells, as indicated. At the end of the 8-h incubation period cultures were washed twice and media replaced with culture media containing IL-1 (100 U/ml). Incubation was for 4 h, after which supernatants were collected to quantitate PGE_2. The media in control cultures were changed at the same intervals as the experimental cultures.

injection of IL-1 to initiate neutrophil infiltration, the circulating neutrophil population was labeled with [^{3}H]thymidine (15). Significant reduction of IL-1-stimulated neutrophil infiltration was observed as early as 48 h, or two injections of mS-oligo-1. After three daily injections of mS-oligo-1, IL-1-stimulated neutrophil infiltration was markedly reduced (Table III). In all these experiments, control injections, vehicle or hS-oligo-1, were without effect. Lack of effect of hS-oligo-1, which contains a 5-base mismatch to the murine sequence, correlated well with results obtained in vitro described above.

Discussion

Antisense oligodeoxynucleotides are an attractive potential method to inhibit expression of cell-surface receptors and attenuate cellular responsiveness. Few reports exist that use synthetic oligomers to target receptors in cells. Synthetic antisense RNA to the subunit of the nicotinic acetylcholine receptor inhibited the expression of receptor-activated currents when coinjected with *Torpedo* mRNA in *Xenopus* oocytes (22). Antisense RNA truncated to cover more 5′ sequences, however, was less effective than full-length RNA. A single oligodeoxynucleotide directed to 5′ sequences also inhibited expression of receptors when it was premixed with *Torpedo* mRNA and coinjected into oocytes. A more recent study found that antisense oligodeoxy-nucleotides directed against the γ- and β-chains of the

Table II. Liposome Delivery of S-oligos to Human Dermal Fibroblasts: IL-1 Receptor Expression

Condition	Specific Binding of [^{125}I]-IL-1
	dpm/well
Basal receptor turnover	
Basal	657±62
Receptor downregulation/acclerated receptor reappearance	
IL-1 18 h, wash	245±30
IL-1 18 h, wash, 4 h recovery	562±46
IL-1 18 h, wash, 4 h recovery with hS-oligo-1	585±60
IL-1 18 h, wash, 4 h recovery with empty liposome	560±38
IL-1 18 h, wash, 4 h recovery with liposome hS-oligo-1	205±16

Data are mean±SEM of triplicate wells for each condition. Human dermal fibroblasts grown to confluency in serum-free media in 96-well plates were exposed to IL-1, 100 U/ml, for 18 h. The cultures were then washed four times with media to remove IL-1 and incubated in IL-1–free media for 4 h. Binding was performed at the times indicated.

murine T cell receptor were effective in suppression of recovery of receptor-mediated function in T cell hybridomas that had been trypsinized to remove existing surface receptors (23).

Since IL-1 receptor-mediated responses are implicated in the pathogenesis of certain inflammatory diseases, we wished to study the effects of antisense oligodeoxynucleotides on the expression and functional activation of IL-1 receptors in cells under conditions more suitable to assess their potential for antiinflammatory action both in vitro and in vivo.

We have demonstrated that oligodeoxynucleotides antisense to the IL-1 receptor block the expression of IL-1 receptors and the biological effects of IL-1 on cultured cells. The degree to which antisense oligonucleotides inhibited IL-1 stimulated PGE_2 synthesis in cultured fibroblasts varied with several factors. O-oligos, which are sensitive to degradation by nucleases, were inactive in serum-containing medium. In serum, which had been heated to inactivate nucleases, the effect of O-oligos was comparable to nuclease-resistant S-oligos with two distinctions: while the inhibition of IL-1 responsiveness occurred more rapidly, it was also more transient. Our results are consistent with the observations that the specific transport of O-oligos across cell membranes occurs rapidly, in contrast to S-oligos which may have greater affinity for the transporter and are released more slowly into the cell interior (11). Whether the more transient inhibition observed with O-oligos was the consequence of an induction of nuclease activity remains to be determined.

Table III. mS-oligo-1 Inhibits IL-1–induced Neutrophil Infiltration in Mice

Condition	Vehicle	mS-oligo-1	hS-oligo-1
		[^{3}H]Tdr, dpm	
One injection	4658±1135	4425±1238	4534±1286
Two injections	5082±720	3512±410*	4825±602
Three injections	5145±918	3242±484‡	4795±1486

* $P = 0.079$, ‡ $P = 0.017$, compared to vehicle, using Student's t test for unpaired observations. Mice received subcutaneous injections of hS-oligo-1 or mS-oligo-1,3 nmol (25 μl), or saline, daily into the same sites for one, two, or three injections. The day before IL-1 was to be administered, animals received [^{3}H]thymidine i.p. to label circulating neutrophils (see Methods). Recombinant human IL-1α, 1,000 U, was injected into each site. After 4 h, animals were killed and punch biopsies were taken. Data are mean±SEM from eight animals. Radioactivity (< 1,000 dpm) in punch biopsies from sites injected with saline alone and not treated with IL-1 have been subtracted from each point.

Size is an important consideration in the design of antisense oligonucleotides. In subcellular preparations, it has been found that longer antisense oligonucleotides are more effective than shorter ones, presumably because binding is more thermodynamically favorable between longer complementary strands (8, 24). In studies using intact cells this phenomenon is far less apparent due to the inefficient transport of long oligodeoxynucleotides into cells (22, 25). We chose 18 mers since this size should yield absolute theoretical specificity for a single mRNA (26) while being still short enough to be transported into cells.

In addition to length, target sequences of antisense oligonucleotides play an important role in their biological efficacy. In general, antisense oligonucleotides directed to 5′ regions of eucaryotic mRNA, particularly near the initiation codon sequences, are more effective than those directed to more 3′ regions (8, 24). While in general agreement, our studies have extended these findings to show that even closely adjacent target sequences yield antisense oligonucleotides with strikingly different potencies. The most inhibitory murine S-oligo (mS-oligo-1; IC_{50} 3 μM), directed to the initiation codon, was 10-fold more potent than mS-oligo-2, directed some 20 bases downstream. The low micromolar potency observed in murine fibroblasts agrees well with that observed for S-oligos in a number of studies (8). Moreover, strict species specificity of inhibition was observed even though base mismatches varied from only two to five bases between murine and human IL-1 receptor sequences. Whether the inhibitory effects of antisense oligonucleotides are attributable to the degradation of mRNA by an RNase H-like activity in cells (27) or to translational arrest (28) it seems likely that local secondary structure of mRNAs plays a critical role in the biological efficacy of these agents.

In resting human dermal fibroblasts the antisense oligonucleotide only poorly inhibited IL-1 receptor expression after 48 h incubation. However, delivery of antisense oligonucleotides by encapsulation in liposomes and fusion to cells markedly increases their inhibitory potential, to essentially 100%. In our studies, however, we found this only to be true under conditions where new receptor synthesis predominantly accounted for cellular responsiveness. Thus protein turnover plays an important role in the consideration of the use antisense agents in the inhibition of gene expression. The results obtained in the downregulation study with human dermal fibroblasts are consistent with an 11-h half-life for IL-1 receptors determined using IL-1 binding in cycloheximide-treated T lymphocytes (19).

Evidence that IL-1 plays a major role in the mediation of many chronic inflammatory diseases, including rheumatoid arthritis (1), has generated great interest in developing agents which inhibit the biological effects of this cytokine. Approaches being explored include drugs that block secretion of IL-1 from inflammatory cells (29–31), use of a soluble, recombinant extracellular binding domain of the IL-1 receptor to bind and

deplete circulating cytokine (32), and competitive antagonism of the IL-1 receptor (33–35). Several proteins that compete with IL-1 for its receptor have been cloned (34, 35); at least one is being investigated as a potential therapeutic agent.

Antisense oligonucleotides present an attractive therapeutic approach predominantly due to their nonpeptidic nature and potential to be absolutely specific. Because the effect of IL-1 in skin is highly localized to the site of injection this was an attractive model in which to test the ability of antisense oligonucleotides to inhibit IL-1 receptor-mediated responses. Of great interest, therefore, was the observation that antisense oligodeoxynucleotides directed against IL-1 receptors blocked IL-1 stimulated neutrophil infiltration in vivo, one of a number of cellular responses in the complex cytokine-mediated inflammatory cascade in mouse dermal tissue. Injection of IL-1 into skin is associated within a few hours with infiltration of neutrophils into the site (20, 21) without edema (21). This effect of IL-1 is a consequence of the activation of type I IL-1 receptors on endothelial cells and subsequent increase in the number of adhesion molecules expressed by these cells (21, 36, 37). It is equally likely that activation of IL-1 receptors increases synthesis of chemotactic eicosanoids by connective tissue cells. These cells would be a primary target of the murine antisense oligonucleotides employed in our studies. It is unlikely that IL-1 receptors on neutrophils would have been affected as these cells are thought to exclusively express type II IL-1 receptors which have been recently demonstrated by cloning to share only ~ 28% amino acid homology to the type I receptor (38). In addition, it is unlikely that intradermal antisense oligonucleotide would have significant access to neutrophils in the blood. While future studies are underway to identify the cell types in mouse dermal tissue that were targets of antisense oligonucleotides to the IL-1 receptor, this is the first report of the successful application of these agents in vivo in a system with potential therapeutic application.

Many hurdles remain, however, before the therapeutic potential of antisense oligonucleotides can be reduced to practice. Present synthetic methodologies are expensive and limited data exist on the in vivo pharmacokinetics and bioavailability of these molecules (39). Speculating that the dose-response data presented in these studies can be translated to an adult human, and, assuming that the volume of distribution of an S-oligo is equal to plasma volume, then a single dose of 50 mg of hS-oligo-1 should be sufficient to reach an effective plasma concentration, thereby making antisense oligonucleotides comparable to many other classes of therapeutic agents. Of course, use of specialized delivery vehicles, such as targeted liposomes, may significantly reduce the required dose and potentially enhance biological effectiveness.

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SPECIFIC INHIBITION OF LYMPHOKINE BIOSYNTHESIS AND AUTOCRINE GROWTH USING ANTISENSE OLIGONUCLEOTIDES IN Th1 AND Th2 HELPER T CELL CLONES

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The helper/inducer class of T lymphocytes has been recently divided into two nonoverlapping subsets on the basis of their lymphokine secretion and requirement for growth (1–3). Helper clones from the Th1 subset produce IL-2 upon antigen or lectin stimulation, whereas clones from the Th2 subset produce IL-4, also known as B cell stimulating factor 1 (BSF-1).[1] Both subsets respond to IL-2, and both express, with minor quantitative differences, receptors for both lymphokines (4). But antibodies directed against IL-2 inhibit only the antigen-stimulated growth of Th1 cells, whereas antibodies directed against IL-4/BSF-1 inhibit only the growth of Th2 cells (4). To confirm this classification, we have used a novel approach, which allows the specific inhibition of the biosynthesis of each respective lymphokine. Antisense inhibition of specific protein synthesis is achieved by saturating the cell with a nucleotide whose sequence is complementary to a portion of the mRNA encoding the protein (5–10). The intracellular annealing of the complementary sequence to the message prevents the synthesis of the protein. The complementary sequence can be introduced into the cell by transfection of a plasmid in which the gene encoding the protein is in opposite orientation with respect to the promoter (6–10). In that case, an intracellular excess of antisense RNA is obtained, and an intracellular RNA/RNA complex is formed. Alternatively, chemically synthesized short complementary DNA sequence can be used (11–19). These synthetic oligonucleotides actively penetrate into the cells, achieving relatively high intracellular levels (19). The advantage of using such a strategy for lymphocytes is that, besides the fact that the low efficiency transfection step can be avoided, the resultant intracellular duplex is a DNA/RNA hybrid. Such duplexes are substrates for an enzymatic activity, RNase H, which

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[1] *Abbreviation used in this paper:* BSF, B cell stimulating factor.

specifically degrades the RNA strand in a DNA/RNA duplex (20–22). Therefore, the efficiency of the inhibition is potentially enhanced.

In this study, we have used two synthetic oligonucleotides, complementary to the 5′ end of the mRNA coding for mouse IL-2 and mouse IL-4 (BSF-1). These sequences correspond to portions of the leader sequence of the molecules. Proliferation of cells from a Th1 clone (D1.1) was inhibited by the IL-2 antisense oligonucleotide, whereas cells from a Th2 helper clone, D10 (23), were only inhibited by the IL-4 antisense oligonucleotide. The inhibition was reversed by addition of exogeneous lymphokine in both cases. The inhibition was accompanied by a decrease in the steady-state level of the corresponding lymphokine mRNA, suggesting a possible degradation of the message through an RNase H like activity.

Materials and Methods

Cell Lines. The D1.1 cell line (2) is a helper T cell from the Th1 subset. It was maintained in 10% FCS RPMI supplemented with 1% glutamine and antibiotics (Gibco Laboratories, Grand Island, NY) and 1% nonessential amino acids (Gibco Laboratories), and stimulated weekly with 100 μg/ml of rabbit IgG presented by irradiated (1,200 rad) BALB/c spleen cells depleted of red cells by a short hypotonic shock. The D10.G4.1 (D.10) (23) cell line is a helper T cell from the Th2 subset. It was maintained in RPMI 10% FCS supplemented with 1% glutamine and antibiotics and stimulated weekly with Conalbumin (100 μg/ml) and mitomycin C–treated C3H/HeJ spleen cells.

Oligonucleotides. Oligonucleotides IL-2 antisense (5′-CTGCATGCTGTACAT-3′), IL-4 antisense (5′-GGGGTTGAGACCCAT-3′), and IL-4 sense (5′-ATGGGTCTCAACCCC-3′) were synthesized on a multiple column DNA synthesizer (model 8700; Biosearch, San Rafael, CA) purified on acrylamide/urea gels followed by electro-elution and several cycles of ethanol precipitation. Oligonucleotides were finally resuspended in PBS before use.

Proliferation Assays. Cells were seeded in microtiter plates in RPMI supplemented as indicated above and with 1% FCS (65°C heat inactivated) and in the presence or absence of various doses of oligonucleotides. After 2 h of incubation at 37°C, the stimulant was added (100 μg/ml of rabbit IgG plus irradiated APCs for D1.1; 100 μg/ml Conalbumin plus mitomycin C–treated APCs for D10) or not for the control, and the concentration of serum was raised to 5%. After a period of 3 d, cultures were washed with RPMI in order to avoid any nonspecific inhibition due to the possible presence of free thymidine from degraded oligonucleotide (three times in 250 μl followed by centrifugation for 3 min at 1,200 rpm); 1 μCi of [^{3}H]thymidine (6.7 Ci/mmol; New England Nuclear, Boston, MA) in 10% FCS RPMI was added in each well and cultures were incubated overnight at 37°C. Cultures were harvested on a Skatron AS (Heggtopen, Norway) cell harvester and incorporated radioactivity was quantified by scintillation counting. All assays were carried out in triplicate. For reversal experiments, 10 U/ml of human IL-2 (Cetus Corp., Emeryville, CA), or 200 U/ml of mouse IL-4 (Immunex Corp., Seattle, WA), or up to 50% of crude D10 conditioned supernatant was added after the preincubation with oligonucleotides.

Northern Analysis. D1.1 or D10.G2 cells (15×10^6 in 5 ml of 1% 65°C heat-inactivated FCS/RPMI) were preincubated in the presence or absence of 5 μM antisense IL-2 or IL-4 oligonucleotide, respectively, for 2 h as described above. 10 μg/ml of Con A (D1.1) or 10 μg/ml of Con A plus 1 U/ml of mouse rIL-1 (kind gift of Dr. W. Benjamin, Hoffmann-La Roche, Inc., Nutley, NJ) (D10) was added and the serum concentration was raised to 5%. After an overnight period of incubation, cells were harvested, washed once in PBS, and resuspended in guanidium-isothyocyanate followed by purification of RNA on a standard cesium chloride gradient.

Equal amounts of RNA (15 μg of total RNA) were denatured with 6% formaldehyde for 10 min at 55°C in 1× MOPS (20 mM morpholine propane sulfonic acid, pH 7.1, 5 mM sodium acetate, 1 mM EDTA) and 50% deionized formamide (Fluka Chemical Co., Hauppage, NY), quickly chilled on ice, and size fractionated on 1% agarose gels containing 6%

formaldehyde. After soaking of gels in 20× SSC (1× SSC = 150 mM sodium chloride, 15 mM trisodium sodium citrate, pH 7), RNA was transferred onto nitrocellulose filters (BA 85; Schleicher & Schuell, Inc., Keene, NH) by capillary blotting in 20× SSC using standard procedures (24). Filters were then baked 2 h under vacuo at 80°C.

All probes used in this study were purified inserts, isolated after appropriate restriction on 1% low-melting-point agarose (Bethesda Research Laboratories, Bethesda, MD), ^{32}P-labeled to 2–5 × 10^8 cpm/μg by primer extension using the Polymeraid labeling kit (PLS) and [^{32}P]dCTP (3,000 Ci/mM; Amersham Corp., Arlington Heights, IL), and was purified on G50 columns (Boehringer Mannheim Biochemicals, Indianapolis, IN). The mouse IL-2 probe was a gift from Frank Lee (DNAX Inc., Palo Alto, CA). The mouse IL-4 probe was a gift from K. Arai (DNAX, Palo Alto, CA). The mouse actin probe was a gift from Dr. K. O'Connel, PRI, Frederick, MD; the ribosomal RNA probe was a gift from Dr. D. Radzioch, PRI. Filters were soaked for 2–24 h in prehybridization buffer (5× SSC, 50% formamide, 1× Denhardt's solution, 20 mM Tris, pH 7.4, 0.2 mg/ml salmon sperm DNA, 5% dextran sulfate), and hybridized with 5–10 ng/ml of labeled probes in the same medium for 20–48 h at 42°C. Filters were washed several times in 2× SSC, 0.1% SDS at room temperature, one to three times 15 min in 0.1× SSC, 0.1% SDS at 50°C, and exposed to Kodak XAR-5 films with Cronex lighting plus intensifying screens (Dupont Co., Wilmington, DE) at −70°C for 3–48 h. For relative quantities of hybridized radioactive probe, autoradiographs were scanned using a densitometer and the relative intensities of the bands were estimated by weighing the densitometer profiles. Hybridized radioactive probe was then removed by immersion of the nitrocellulose filters in boiling water. Filters were checked before use with other probes.

Results

Specific Inhibition of the Proliferation of Th1 or Th2 Cell Lines by IL-2 or IL-4 Antisense Oligonucleotides. Previous studies by our laboratory and others (16–19) have shown that short oligonucleotides readily penetrate lymphoid or myeloid cells, reaching significant levels within a few hours. Therefore, cells from the D1.1 clone, a murine Th1 helper clone (1) or from the D10 clone, a murine Th2 helper clone (23), were preincubated for 2 h with various doses of anti-IL-2 or anti-IL-4 antisense oligonucleotides, and activated with antigen and irradiated APCs. Proliferation was measured at day 3, using a thymidine incorporation assay as described in the Materials and Methods. D1.1 cell proliferation was inhibited by the anti-IL-2 antisense oligonucleotide, whereas D10 cells were inhibited by the anti-IL-4 oligonucleotide (Fig. 1). In both systems, a maximal inhibitory effect (90–100%) was reached between 5 and 10 μM of oligonucleotide. In both systems, the nonhomologous oligonucleotide against the reciprocal interleukin did not have such an antiproliferative effect, indicating that the inhibition was specific and not due to a toxicity of the oligonucleotides.

The Inhibition Can Be Specifically Reversed by the Addition of Exogeneous Lymphokine. The specificity of the inhibition was further assessed by reversal experiments. We reasoned that if the inhibition was due to the lack of lymphokine biosynthesis, the inhibition should be reversed by the addition of exogenous IL-2 or IL-4. Therefore, antisense-treated D1.1 or D10 cells were cultured in the presence or absence of exogeneously added IL-2 or IL-4, respectively. The results shown in Fig. 2 indicated that exogeneous rIL2 was able to significantly reverse the proliferative blockade of antisense IL-2-treated D1.1 cells, further demonstrating that the inhibition was specific and not due to a toxic effect of the oligonucleotide. Similarly, exogeneously added rIL-4 was able to overcome the inhibition of D10 proliferation by antisense IL-4

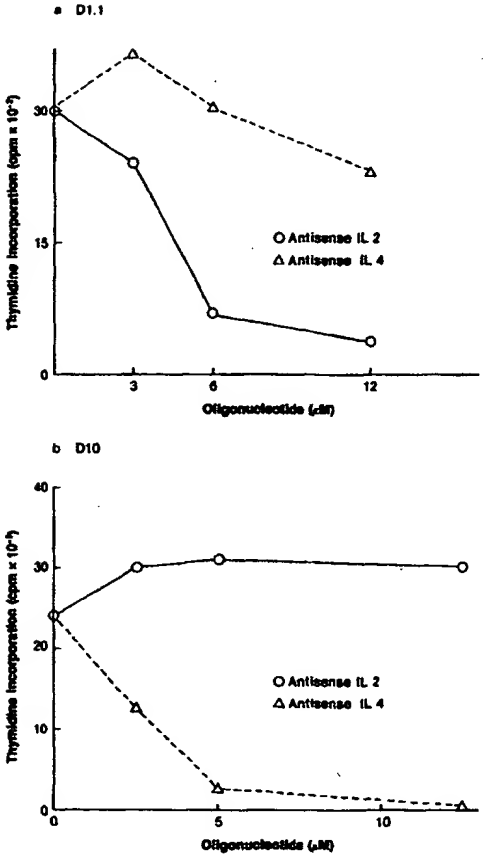

FIGURE 1. Inhibition of Th clones proliferation by antisense oligonucleotides. D1.1 (*a*) or D 10 (*b*) cells were incubated 2 h with indicated doses of IL-2 or IL-4 antisense oligonucleotides, activated with antigen plus APCs, and thymidine pulsed 3 d later. The figure shows the result of a typical experiment, and three to five independent experiments gave the same results.

oligomer. However, in the case of the D10 cells, this reversal effect was observed only in the low range of oligonucleotide concentration. A partial reconstitution was also observed when a crude supernatant from antigen-activated D10 cells was used instead of rIL4 (data not shown). This suggests that this lack of complete reconstitution was not related to an intracellular induction by IL-4 of other indispensable growth factors that could be present in the crude supernatant. A sense control oligonucleotide did not have any effect on D10 cell proliferation in the range of doses used for the antisense (data not shown). It is likely that, at high doses, the antisense IL-4 oligonucleotide caused some nonspecific inhibition. This is confirmed by the slight nonspecific inhibition observed on D11 cells using the antisense IL-4 oligonucleotide, and could be related to the sequence itself.

Treatment with the Antisense Oligonucleotides Results in Reduced Steady-State Level of Lymphokine Message. To gain insight into the mechanism by which the antisense oligonucleotides inhibited the autocrine growth of D1.1 and D10 cells, we performed Northern analysis of the steady-state levels of lymphokine messages in these cells. D1.1 or D10 cells were incubated with antisense IL-2 or IL-4 oligonucleotides, respectively, and activated with Con A (D1.1) or Con A plus IL-1 (D10). After an overnight

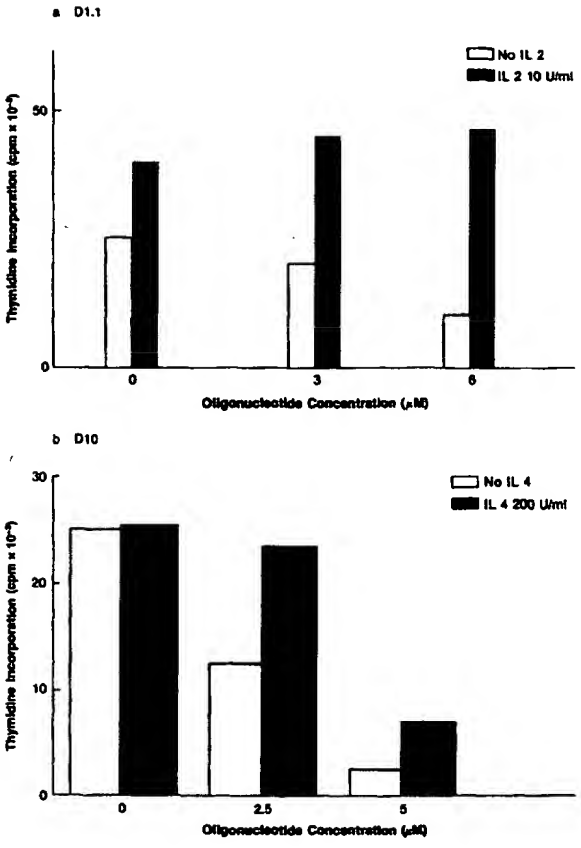

FIGURE 2. Reversal of inhibition by exogenous lymphokines. D1.1 (*a*) or D10 (*b*) cells were incubated with or without IL-2 or IL-4 antisense oligonucleotides as indicated, and activated with antigen and APCs in the presence or absence, as indicated, of exogenous lymphokine. Cultures were thymidine pulsed 3 d later. The figure shows the result of a typical experiment, and two to three independent experiments gave identical results.

period of culture, cells were lysed and total RNAs were extracted and analyzed using IL-2 and IL-4 cDNA probes (Fig. 3). The antisense IL-2-treated D1.1 cells did not express detectable level of IL-2 message, when compared with untreated controls similarly activated with Con A plus IL-1. Conversely, the antisense IL-4-treated D10 cells showed a significantly decreased level of IL-4 message (fivefold decrease). No IL-4 message was detected in any of the D1.1 RNA samples and no IL-2 message was detected in any of the D10 RNA samples (data not shown). The decrease in lymphokine message was specific, since the steady-state level of actin message was not impaired by the treatment with oligonucleotides (Fig. 3). Furthermore, the level of rRNA was also unchanged in these samples (data not shown).

Discussion

In procaryotic cells, specific blockade of the expression of a gene has been one of the major tools to assess the function of its product, through the use of deletion or thermosensitive mutants. In mammalian cells, the difficulty of deriving mutant cell line has prompted the search for other means of specific gene product deletion. A possible solution was derived from the study of gene regulation in bacteria, where

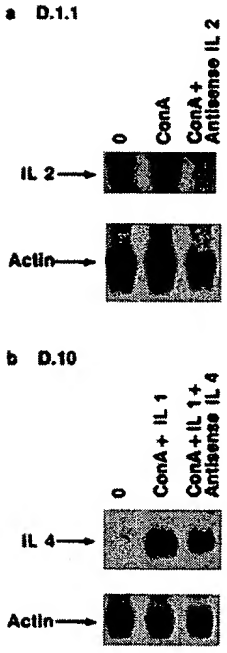

FIGURE 3. The steady-state level of lymphokine mRNA is decreased in the presence of antisense oligonucleotides. D1.1 (*a*) or D10 (*b*) cells were incubated with or without IL-2 or IL-4 antisense oligonucleotides as indicated, and activated with Con A or Con A plus IL-1 as indicated. After an overnight period of culture, cells were washed and RNAs were extracted and purified. RNAs were electrophoresed on denaturing agarose gels, blotted, and analyzed for the level of IL-2, IL-4, or actin mRNA, as indicated.

the expression of certain genes is regulated by naturally occurring complementary RNAs: such antisense RNAs are thought to hybridize with the normal message and therefore to block its recognition and processing by the translational apparatus of the cell (reviewed in reference 5).

Recent studies have shown that artificial introduction of antisense plasmids into eukaryotic cells resulted in a specific inhibition of the expression of exogenous as well as endogeneous cellular genes (6–10). The caveats of this technique are that: (*a*) One needs to be able to efficiently introduce exogenous DNA in the cells; (*b*) There is a general requirement to achieve a 100-fold excess of the antisense over the endogeneous sense message; and (*c*) In the specific case of genes related to cell proliferation, an inducible blockade is required. Inducible promoters such as the dexamethasone-inducible MMTV promoter, have been successfully used in mouse fibroblasts for specific inhibition of the c-*fos* gene (10). This promoter, however, cannot be universally used, since dexamethasone is, by itself, inhibitory for growth of most lymphoid cells. Alternatively, synthetic oligonucleotides complementary to viral genes have been shown to prevent infection of cells by several viruses, without affecting normal cell growth (11–15). In addition, we and others (16–19) have specifically deleted the expression of c-*myc* gene in T lymphocytes or in myeloid cells. In the present study, we demonstrate the successful use of this strategy for the selective deletion of secretory proteins in lymphocytes.

Studies of the lymphokine production and requirement for growth of cloned helper T cell have shown the existence of two subsets, the Th1 and the Th2 subset. Using antibodies directed against these two lymphokines, it has been demonstrated that

the Th1 type produces IL-2 and proliferates using IL-2 as an autocrine factor, whereas the Th2 type helper clones produces IL-4 and not IL-2, and proliferates using IL-4 as an autocrine factor. The two subsets are not overlapping, the Th1 type helper clones being inhibited only by anti-IL-2 antibodies, whereas the Th2 type helper clones are inhibited only by anti-IL-4 antibodies. We here confirm these observations with the antisense strategy, using complementary, or antisense, short oligodeoxynucleotides. We show that antisense directed against the IL-2 message was able to inhibit only a Th1 helper clone proliferation, whereas antisense directed against the IL-4 message inhibited only cells from a Th2 clone. The inhibition was not due to a toxic effect of the oligonucleotides, since no inhibition was observed with the antisense IL-4 on IL-2-dependent T cells, and vice-versa.

Furthermore, the inhibition could be reversed by exogenous addition of the lymphokine, although in the case of the antisense IL-4 oligonucleotide at high doses, the reversal was only partial. This could suggest an intracellular effect of the lymphokine that could not be reproduced by the addition of extracellular IL-4. It is likely, however, that this partial reversal reflects a nonspecific inhibition by high doses of antisense IL-4, which is not detected with the antisense IL-2, but has been observed in various systems.

The antisense oligonucleotides were originally thought to act through a specific inhibition of mRNA translation, on the basis of the results of several in vitro studies (25–28). However, it has recently been suggested that in certain systems, the protein synthesis inhibition could in fact result from the degradation of the relevant mRNA by an enzymatic activity known as RNAse H (22). RNAse H specifically degrades the RNA strand in a DNA/RNA duplex. In this study, we show that the inhibition of cell proliferation was accompanied by a lower steady-state level of the relevant message in each cell line. It is not known, at this point, if this lower level of message results from a transcriptional or post-transcriptional event. It could be due to a feedback effect of the disappearance of the protein, as it has been shown with mutated globin genes, for example (29). However, a likely hypothesis is that the lymphokine messages are degraded by an RNase H-like activity.

This type of study, involving the actual and specific inhibition of a lymphokine, could prove helpful in understanding the respective role of each of the helper T cell subsets in the different immunological situations in which helper T cells are known to be involved. Antibody-mediated inhibition of lymphokines often does not give consistent results. Antibody inhibition relies on the fact that the antibody can inhibit the binding of the lymphokine to the cellular receptor. The inhibition therefore depends on the relative affinity of the antibody versus the physiological ligand for the receptor. Furthermore, no effect would be obtained on the potential intracellular action of the lymphokine in the producing cell. The antisense oligonucleotide strategy, which allows the inhibition of the actual biosynthesis of the lymphokine, could overcome these difficulties. In addition, antisensing lymphokines could have potential therapeutic applications in the case of autocrine cancer cells, as it is already envisioned in the case of viral disease (11–15).

Summary

T helper cells have recently been divided into two subsets. The Th1 subset secretes and responds to IL-2 in an autocrine manner. The Th2 subset upon mitogen

or antigen stimulation releases IL-4. Here we describe a novel technology that allowed us to confirm this distinction. We have used synthetic oligonucleotides complementary to the 5' end of mouse IL-2 and IL-4 to specifically block the biosynthesis of IL-2 or IL-4 in two murine helper T cell clones from the Th1 or Th2 subset. We show that the antisense IL-2 oligonucleotide inhibited the proliferation of the Th1 clone and had no effect on the Th2 clone. In parallel experiments, the antisense IL-4 oligonucleotide blocked the proliferation of the Th2 clone and not the proliferation of the Th1 clone. The inhibition was significantly reversed in both cases by the addition of the relevant lymphokine (IL-2 in the case of the Th1 clone, IL-4 in the case of the Th2 clone). Northern analysis, using cDNA probes specific for the two lymphokines, showed a decrease in the steady-state level of the relevant lymphokine mRNA, suggesting the specific degradation of the mRNA by an RNase H-like enzymatic activity.

This strategy, which allows the specific blockade of the biosynthesis of a lymphokine, could be useful for future studies on the role of each T helper subset in physiological immune responses.

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Use of an Epstein–Barr virus episomal replicon for anti-sense RNA-mediated gene inhibition in a human cytotoxic T-cell clone

(CD8)

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ABSTRACT **A methodology was developed for stable gene transfer into cloned nontransformed human T lymphocytes. Stable high-level gene expression was achieved in cloned human T cells by using a self-replicating Epstein–Barr virus (EBV) episomal replicon. A comparison of five eukaryotic promoters established that the Rous sarcoma virus 3′ long terminal repeat (RSV 3′ LTR) and the lymphopapilloma virus (LPV) 5′ LTR are optimal for episome-based expression in T cells. Effective (>95%), selective, and reversible anti-sense RNA-mediated gene inhibition of a model T-cell-associated molecule (CD8) was achieved in a cytotoxic human T-cell clone by using an EBV episome-based, RSV 3′ LTR-driven expression system. The linking of anti-sense RNA mutagenesis and T-cell cloning technologies should contribute significantly to studies of human T-cell function.**

Gene transfection offers a powerful experimental approach for defining the functional roles of specific molecules in T lymphocytes. Stable gene transfer has been accomplished for T-cell tumor lines (1–4), T-cell hybridomas (5, 6), cord blood lymphocytes (7), and cloned murine T lymphocytes (8–10) but has not been described for cloned human T lymphocytes. Unlike T-cell tumor lines and hybridomas, T-cell clones possess normal karyotypes; are regulated in their proliferation by antigen, lymphokine secretion, and lymphokine receptors; and readily mediate specific and nonspecific cytotoxicity (11). Hence, the availability of transfection mutants of cloned, human T-lymphocyte lines would contribute significantly to molecular studies of human T-cell function.

Plus-sense transfection analysis has been applied to the study of a variety of T-cell-associated molecules such as the T-cell antigen receptor (5), the interleukin 2 (IL-2) receptor (8), Thy-1.2 (1), CD8 (6), CD4 (12), CD7 (13), and interferon-γ (9). However, the anti-sense RNA mutational approach (14) has not been used in studies of T lymphocytes. Anti-sense RNA technology, by enabling selective gene inhibition, provides an alternative transfection strategy in which deletional mutants can be used to determine whether specific molecules play obligatory roles in defined cellular functions.

Our goal in the present study was to develop an efficient system for generating anti-sense RNA mutants of cloned human T lymphocytes. To this end, we have explored the utility of a high copy number Epstein–Barr virus (EBV) episomal replicon (15) to serve as a vector for high level stable expression of sense and anti-sense RNA transcripts. Episomal replicons are circular DNA elements designed to self-propagate extrachromosomally in eukaryotic cells. As expression vectors, episomal replicons offer a means for amplifying gene copy number in cells and, furthermore, circumvent complications that arise from chromosomal integration—e.g., position effects on levels of transcription and insertional mutagenesis of host cell sequences at the integration site. The EBV origin of replication (oriP) and the EBV nuclear antigen 1 together confer an episomal replication capacity to circular DNA elements in an array of human cell types, including lymphoid cells (15). Here we describe a methodology for stable gene transfer into cloned nontransformed human T lymphocytes by using an EBV episomal replicon, and we identify promoters suitable for episome-based expression in these cells. In addition, we establish the utility of episomal vectors for anti-sense RNA-mediated gene inhibition of a model surface glycoprotein, CD8, on a human cytotoxic T-cell clone.

MATERIALS AND METHODS

Reagents. Complete medium consisted of RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (HyClone, Logan, UT), L-glutamine (2 mM), Hepes (10 mM), penicillin (5 units/ml), and streptomycin (5 μg/ml). Recombinant DNA enzymes were obtained from New England Biolabs, Boehringer Mannheim, and Pharmacia. Other reagents were obtained from the indicated sources: chloramphenicol and lysozyme (Sigma); polyethylene glycol 1540 (Koch Light); hygromycin B and A23187 (Calbiochem; A23187 stock solution: 0.7 mM in ethanol); [^{14}C]chloramphenicol (New England Nuclear); acetyl CoA lithium salt (Pharmacia).

Cells. Two independently derived nontransformed (IL-2 dependent) human T-cell clones, V1 ($CD4^+CD8^-$) and 8L2 ($CD4^-CD8^+$), were used for these studies. The derivation of the influenza hemagglutinin-specific HLA-DR5-restricted cytotoxic cell line V1 has been described (16, 17). The 8L2 line was cloned by micromanipulation of single cells (Autoclone, EPICS V, Coulter) from mixed lymphocyte cultures in 96-well microtiter plates containing 200 μl of complete medium per well. To each well we added 20,000 γ-irradiated (5000 rads; 1 rad = 0.01 Gy) allogeneic human peripheral blood mononuclear cells (PBMC) and 10% MLA-144 culture supernatant (CS) as a source of IL-2. The growth cycle of both of these clones is regulated and characterized by transient expression of IL-2 receptors (16). Clones were stimulated weekly with OKT3 (anti-CD3) monoclonal antibody (mAb; 1 ng/ml; Ortho Diagnostics), IL-2 (10% MLA-144 CS), and irradiated allogeneic human PBMC (0.75×10^6 cells per ml). After 3 days of stimulation, clones were washed

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Abbreviations: EBV, Epstein–Barr virus; RSV 3′ LTR, Rous sarcoma virus 3′ long terminal repeat; LPV, lymphopapilloma virus; IL-2, interleukin 2; oriP, EBV origin of replication; PBMC, peripheral blood mononuclear cells; CS, culture supernatant; mAb, monoclonal antibody; CAT, chloramphenicol acetyltransferase; PHA, phytohemagglutinin.

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and subcultured in fresh complete medium supplemented with IL-2 (10% MLA-144 CS) for 4 days prior to restimulation (Fig. 1).

Plasmid Constructions. To assemble promoter–chloramphenicol acetyltransferase (CAT)/220.2 plasmids (Fig. 2*a*), we obtained promoter–CAT plasmids from several investigators: SV2CAT (simian virus 40 early promoter; B. Howard; ref. 18); RSVCAT [Rous sarcoma virus 3′ long terminal repeat (RSV 3′ LTR); B. Howard; ref. 19]; LPV-5′-CAT [lymphopapilloma virus (LPV) 5′ LTR; R. Miksicek; ref. 20]; I10CAT (rat GRP78 gene calcium ionophore-inducible promoter; A. Lee; ref. 21); HS1CAT (phMT-II$_A$-CAT; human heavy metal-inducible metallothionein II$_A$ promoter region encompassing ≈850 base pairs of sequence upstream of the hMTII$_A$ gene; M. Karin and A. Haslinger; ref. 22); and SV0CAT (no eukaryotic promoter; B. Howard; ref. 18). p220.2 was generously provided to us by B. Sugden (Madison, WI). This 8.9-kilobase plasmid is a derivative of p201 (15) in which a polylinker (*Bam*HI, *Xba* I, *Sal* I, *Pst* I, *Hin*dIII) has been inserted at the *Nar* I site within the herpes simplex virus 1 thymidine kinase gene termination sequence (B. Sugden, personal communication; see Fig. 2*a*). For construction of promoter–CAT/220.2 plasmids, a second *Bam*HI site was first introduced at unique restriction sites upstream of the promoter in promoter–CAT plasmids using *Bam*HI linkers (New England Biolabs), and promoter–CAT cartridges were then mobilized by *Bam*HI digestion and inserted in both orientations into the unique *Bam*HI site of p220.2 (C.A.H. and M.L.T., unpublished data).

pT8F1 (generously provided to us by R. Axel; ref. 23), a cDNA plasmid encompassing the complete coding sequence of human CD8, was the source for the CD8 DNA segment in the anti-sense CD8 construct α-CD8/REP1 (see Fig. 3 and *Results*). RSVPA1, a plasmid in which the RSV 3′ LTR (derived from pRSVCAT), *Eco*RV and *Bam*HI subcloning sites, and the simian virus 40 late polyadenylylation/termination sequence (derived from pcDV1; ref. 24) are sequentially arrayed, was assembled by us in a multistep procedure (R.K.G., H.K.S., and M.L.T., unpublished data).

Transfection Procedures. Electroporation (used here for promoter–CAT/220.2 episomes) was performed with a Promega Biotec X-Cell 450 electroporator at 200 V, 1100 μF, 0.7 sec time load, in phosphate-buffered saline (PBS) containing 20 mM Hepes (pH 7.2), 500 μg of sheared salmon sperm DNA as carrier, and 20 μg of plasmid DNA. The protocol for protoplast fusion (used here for the α-CD8/REP1 episome) has been described (25), and we have introduced modifications for suspension cells. Protoplasts were copelleted with 10^7 logarithmically growing target cells (prewashed with PBS three times) (5000:1, protoplast/target cell ratio) at 1700 × *g* in a DuPont-Sorvall HS-4 centrifuge for 20 min at 4°C. Fifty percent polyethylene glycol 1540 in PBS (1 ml) was added dropwise over 1 min, and cells were then immediately diluted with PBS (1 ml) over 1 min and more PBS (20 ml) over 3 min, pelleted, washed twice with PBS, and resuspended in six-well plates (10^6 cells per ml) in complete medium supplemented with gentamicin (200 μg/ml) and IL-2. Stable transfectant lines corresponding to individual wells were derived by the

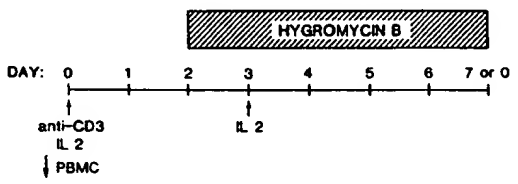

FIG. 1. Stable gene transfer into human T-cell clones. A 7-day cycling scheme was used to derive and maintain cloned human T-cell transfectants. Cells were washed and stimulated at the start of the cycle (day 0) with anti-CD3 (OKT3) mAb, IL-2 (10% MLA CS), and irradiated PBMC, and washed and restimulated (with exogenous IL-2 only) on day 3. Hygromycin B (0.15 mg/ml) was added to the culture medium on day 2 and again on day 3 (after the wash).

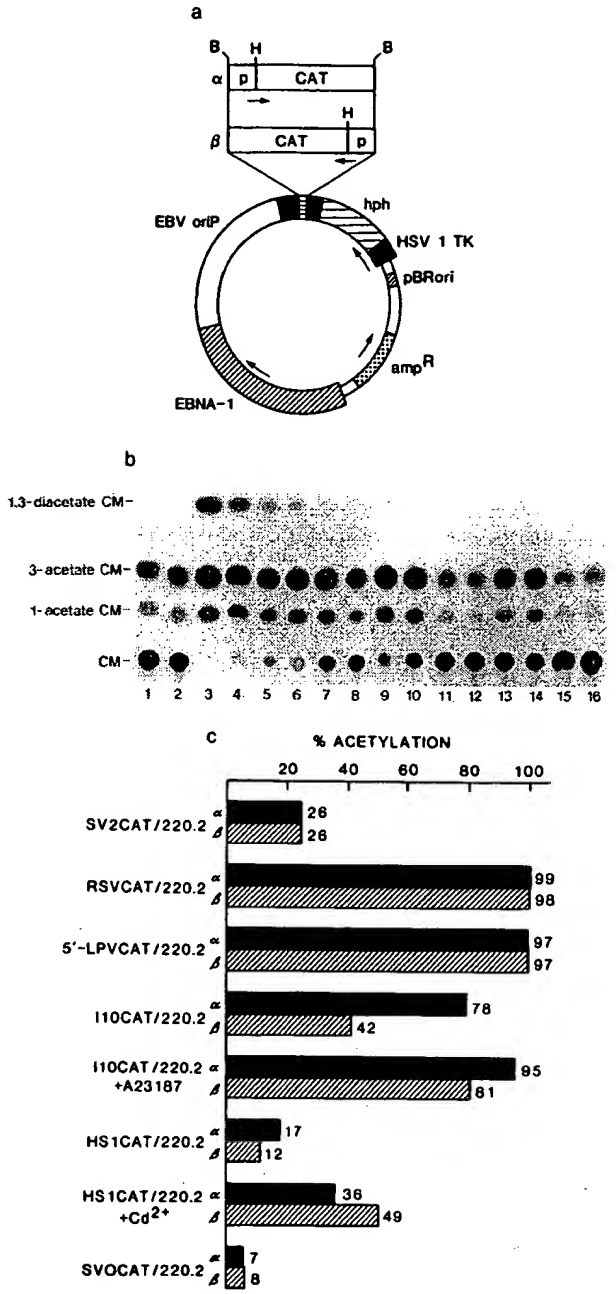

FIG. 2. Stable CAT expression in a human T-cell clone using different episome-based eukaryotic promoter elements. (*a*) Schematic representation of promoter–CAT/220.2 episomes, showing the EBV oriP, a functional segment of the EBV nuclear antigen 1 gene (EBNA-1), the *Escherichia coli hph* gene, herpes simplex virus thymidine kinase 1 (HSV 1 TK) promoter and termination sequences (solid bars), pUC12-derived multiple cloning site sequence (horizontal stripe), pBR322-derived sequences (narrow band), pBR322 origin of replication (pBRori), ampicillin-resistance gene (amp^R), and α- and β-oriented promoter–CAT cartridges. Arrows indicate direction of transcription. B, *Bam*HI; H, *Hin*dIII. (*b* and *c*) CAT activities for various promoter–CAT/220.2 V1 transfectants. TLC autoradiograms (*b*) and % acetylation (*c*) are shown. Numbers 1–16 in *b* correspond to promoter–CAT/220.2 constructs listed (top to bottom) in *c*, in that order. Numbers to right of bars (*c*) indicate % acetylation [1-acetylated + 3-acetylated + 1,3-diacetylated cpm/total (unacetylated + acetylated) cpm].

protocol described in *Results*, and transfectants were not recloned for these studies.

CAT Enzymatic Assay. Cells (5×10^6) were harvested, washed three times in PBS, resuspended in 25 mM Tris (pH 7.8) (100 μl) and lysed by five cycles of freeze–thawing. Crude cellular extracts (20 μl per reaction mixture) were assayed for chloramphenicol acetylating activity by a standard 1-hr assay as described (18). Enzymatic mixtures were extracted with ethyl acetate, and unacetylated and acetylated (1-acetate, 3-acetate, 1,3-diacetate) forms of [^{14}C]chloramphenicol were separated by ascending chloroform/methanol (95:5) thin-layer chromatography on silica gel plates (20 × 20 cm) (Whatman). Spots were visualized by overnight autoradiography (Kodak XAR film; −70°C with intensifying screen) and quantitated by excision of spots and liquid scintillation counting.

Flow Cytometry. Cells were immunostained as described (16) using OKT8 (anti-CD8), OKT3 (anti-CD3), OKT11 (anti-CD2; Ortho Diagnostics), or normal mouse IgG (Miles) as primary antibodies, and fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Miles) was used as the secondary antibody. Cells were analyzed on an Epics V fluorescence-activated cell sorter (Coulter).

RESULTS

For these studies, we used the episomal replicon p220.2 (15), which contains the EBV sequences required for episomal replication in human cells, as well as the *E. coli hph* gene, which confers resistance to the eukaryocidal antibiotic hygromycin B. Since the human T-cell clones require feeder cells (PBMC) which are hygromycin B sensitive, we developed a modified selection protocol to permit the derivation of stable hygromycin B-resistant (hyg^R) transfectants. After transfection by electroporation or protoplast fusion, cells were resuspended in complete medium supplemented with IL-2. Cells were stimulated with IL-2, anti-CD3 antibody, and irradiated PBMC 2 days posttransfection, and hygromycin B was added 2 days later. A 7-day cycling scheme (Fig. 1) was then initiated in which hygromycin B was present in the culture medium only on days 2–7 after stimulation with irradiated fresh PBMC, IL-2, and anti-CD3 antibody. After 5 days of incubation with hygromycin B (0.15 mg/ml), control nontransfected cell cultures contained virtually no viable cells. Stable hyg^R transfectants have been reproducibly obtained (≈50% of transfections) for several cloned T-cell lines by this protocol.

A systematic investigation of promoter function in EBV episomal replicons has not been previously reported. To address this issue, as well as to specifically explore the utility of EBV-based episomal replicons for the expression of transfected genes in human T cells, we assessed episomal replicon-based promoter function in our cloned T-cell line V1. A panel of eukaryotic promoters consisting of various constitutive (simian virus 40 early, RSV 3′ LTR, LPV 5′ LTR) and inducible (*GRP78* gene, *hMTII$_A$* gene) promoters were examined. For these analyses, the prokaryotic CAT gene, which is absent in eukaryotic cells, served as a reporter gene for promoter-driven transcriptional activity (19, 26). Promoter–CAT cartridges were inserted, in both orientations, into the EBV-based replicon p220.2 at the unique *Bam*HI site, which lies just downstream of the EBV oriP (Fig. 2*a*; see *Materials and Methods*). The alternative orientations for cartridges in promoter–CAT/220.2 plasmids have been arbitrarily designated α (promoter is proximal to EBV oriP) and β (promoter is distal to EBV oriP).

Stable hyg^R V1 transfectants for each of the promoter–CAT/220.2 plasmids were independently derived, and the CAT enzymatic activity in them was compared (Fig. 2 *b* and *c*). The RSV 3′ LTR-based episomes yielded maximal CAT activity, which, based on titration experiments (data not shown), was marginally higher than the strong activity seen with the LPV 5′ LTR. In contrast, the episomes incorporating the simian virus 40 early promoter, a constitutive promoter commonly used in eukaryotic expression work (24), were significantly less efficient in driving CAT expression. The inducible GRP78 (I10CAT/220.2) and hMTII$_A$ (HS1CAT/220.2) gene promoters both displayed high levels of basal activity and showed 22% (7 μM A23187) and 93% (10 μM cadmium) induction for α-oriented promoters, respectively, and 112% and 308% induction for β-oriented promoters. However, the levels of CAT activity seen after induction of these promoters were significantly below those for either of the LTR-based (RSV and LPV) constitutive promoters. The orientation dependence of promoter activity ($\alpha > \beta$) seen with some promoters—e.g., the GRP78 gene promoter—may result from a transcriptional enhancer effect exerted by the EBV oriP (27). An episome analogous to RSVCATα/220.2, in which plus-sense CD8 instead of CAT is driven by the RSV 3′ LTR, yielded high levels of surface CD8 on transfectants (data not shown), establishing the utility of episome-based RSV 3′ LTR-driven expression systems for genes encoding cell-surface proteins as well as for cytoplasmic proteins.

To determine whether EBV episomal replicons can be used effectively for stable anti-sense RNA-mediated gene inhibition in cloned T cells, we selected CD8 as a model T-cell surface protein. To inhibit CD8 expression, we constructed the episome α-CD8/REP1 (Fig. 3). A 459-base-pair CD8 coding segment, spanning amino acids 9–161 of the 214-amino-acid-long processed CD8 protein, was first inserted in an inverted orientation downstream of the RSV 3′ LTR and upstream of the simian virus 40 polyadenylylation specification sequence from the late region of the virus. This promoter/α-CD8/polyadenylylation unit was subsequently cartridged in an α-orientation into the EBV episomal shuttle vector p220.2. α-CD8/REP1 was stably transfected into 8L2, a CD4$^-$CD8$^+$ T-cell clone derived by nonspecific stimulation via anti-CD3 mAbs and irradiated allogeneic feeder cells. The antigenic specificity and major histocompatibility complex restriction of this clone are unknown.

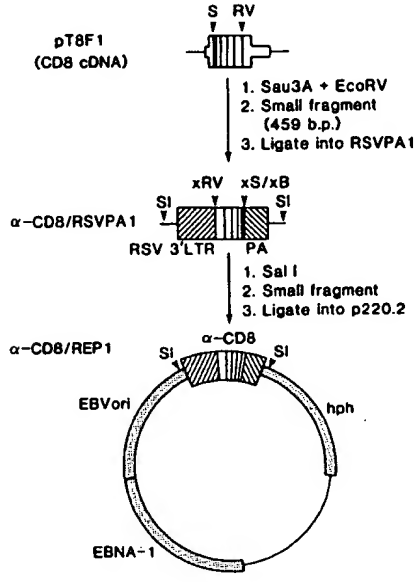

FIG. 3. Assembly of α-CD8/REP1 (anti-sense CD8) episome. Sources for pT8F1, p220.2, and RSVPA1 are described in *Materials and Methods*. S, *Sau*3A; RV, *Eco*RV; SI, *Sal* I. x indicates that the restriction site has been eliminated. PA, simian virus 40 late polyadenylylation/termination sequence; b.p., base pairs.

The level of surface CD8 expression on the parental and transfected 8L2 lines was determined 46 days posttransfection by flow cytometry using an anti-CD8 mAb (Fig. 4). Whereas nontransfected 8L2 and RSVCATα/220.2 8L2-transfected cells displayed equivalent levels of surface CD8, the α-CD8/REP1 8L2 transfectant demonstrated a marked decrease (>95%) in surface CD8 expression. The absence of alterations in the cell-surface expression of CD2 (sheep erythrocyte receptor) and CD3 (a component of the T-cell $\alpha\beta$ antigen receptor complex) in the α-CD8/REP1 transfectant (Fig. 4) established the specificity of this inhibition. Neoexpression of CD4 and CD1, surface molecules that are not expressed on 8L2 cells, was not observed for the CD8-suppressed transfectant (data not shown).

To establish that the anti-sense RNA inhibition was indeed mediated by an EBV-based episome, we transferred an aliquot of α-CD8/REP1-transfected cells into hygromycin B-free medium 39 days posttransfection and followed the stability of the mutant phenotype by serial flow cytometric analyses (Fig. 5). A gradual loss of EBV-based episomes is known to occur after removal of the selective agent (15). As expected, in the absence of hygromycin B, surface CD8 gradually reemerged in a time-dependent fashion, suggesting a progressive loss of the α-CD8/REP1 episome. After 1 week in hygromycin B-free medium, no change in the level of CD8 expression was detectable; after 2 weeks, an increase in CD8 expression was observed; by 5 weeks, surface CD8 levels approached those on the control RSVCATα/220.2 8L2 transfectants. In contrast, when the transfectant was maintained in the continuous presence of hygromycin B, CD8 inhibition remained stable for the entire 3-month observation period. The finding that the CD8-suppressed phenotype is stable for 1 week out of hygromycin B means that this agent can be temporarily cleared from the cells prior to functional assays. Also, the incremental increases in CD8 expression that occur after removal of the selective agent represent an experimental means for exploiting episome-based expression systems for gene dosage analyses.

Functional studies were performed on the α-CD8/REP1 transfectant grown in the continuous presence of hygromycin B. Nonspecific cytotoxicity mediated by either anti-CD3 mAb or the lectin phytohemagglutinin (PHA) did not differ significantly between the CD8 anti-sense mutant and controls (Fig. 6). Moreover, there were no significant differences in the expression of the IL-2 receptor (as detected by anti-Tac mAb) after anti-CD3 mAb stimulation, the proliferative response to either PHA or anti-CD mAb, or the response to phorbol diester as measured by increased IL-2 receptor expression (data not shown).

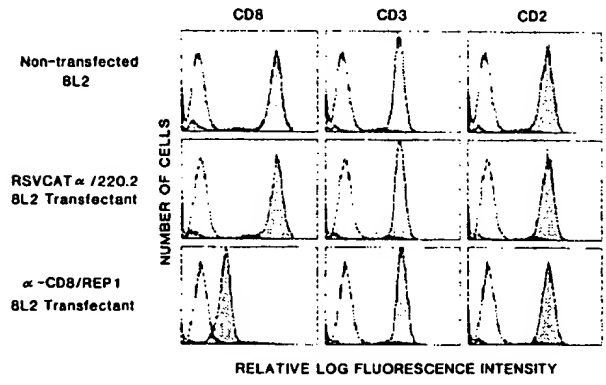

FIG. 4. Selective anti-sense RNA-mediated inhibition of surface CD8 expression on 8L2 cells. α-CD8/REP1 and RSVCATα/220.2 8L2 transfectants and parental 8L2 cells were stained on day 46 with OKT8 (anti-CD8), OKT3 (anti-CD3), and OKT11 (anti-CD2) (stippled areas) or normal mouse IgG (Miles; open areas) as primary antibodies, and fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Miles) was used as the secondary antibody.

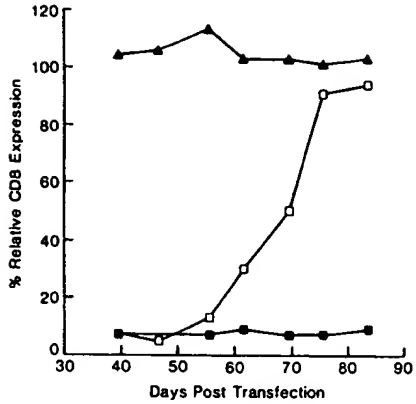

FIG. 5. Reappearance of surface CD8 on α-CD8/REP1 hygromycin B-resistant 8L2 transfectant line after removal of hygromycin B. Stable α-CD8/REP1 (squares) and RSVCATα/220.2 (triangles) 8L2 transfectants were derived as described in text. On day 39, a subculture of the α-CD8/REP1 transfectant (open squares) was washed free of hygromycin B and then maintained in the absence of this selective agent. CD8 expression was assessed at the indicated time points by flow cytometric analysis. Relative expression was calculated by comparing the peak channel number (pcn) for each group with the pcn for nontransfected 8L2 (pcn range, 190–201) at each time point.

DISCUSSION

In this study, we have described a methodology for the derivation and maintenance of stably transfected cloned human T lymphocytes. This entails the use of a 7-day cycling scheme in which the eukaryocidal antibiotic serving as a selective agent is absent for the first 2 days after the addition of irradiated antibiotic-sensitive feeder cells in each cycle.

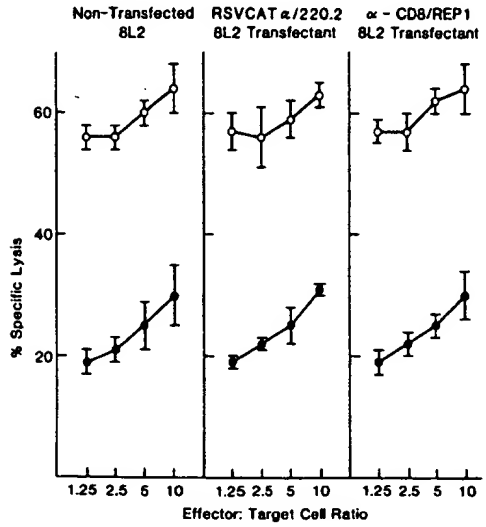

FIG. 6. Anti-CD3- and PHA-mediated cytotoxicity of U937 targets by nontransfected 8L2 cells and stable 8L2 transfectants (RSVCATα/220.2 and α-CD8/REP1). Cytolysis of ^{51}Cr-labeled U937 targets (5×10^3 cells per well) was mediated by either PHA (0.1 μg/ml) (open circles) or OKT3 (2 ng/ml) (solid circles) at various effector target cell ratios in a standard ^{51}Cr release assay (17). Maximal release of ^{51}Cr was achieved in 1% Triton X-100. Spontaneous release in these experiments was 16%. Specific lysis was calculated as described (17). Stable transfectants were selected in hygromycin B for 62 days and washed free of the antibiotic immediately prior to their use in this assay.

This protocol can be used in conjunction with electroporation or protoplast fusion as alternative transfection modalities and yields stable transfectants in a reproducible fashion.

Furthermore, we have shown that stable expression of transfected genes can be achieved in cloned human T cells by using an EBV-based episomal replicon. A comparison of five eukaryotic promoters demonstrated that the RSV 3' LTR and LPV 5' LTR are most suitable for high level EBV episome-based expression in T cells. Differences in CAT activity observed in this episomal expression system may reflect not only variation in promoter activity but also differences in episome copy numbers per cell, since it is conceivable that particular promoter sequence elements may influence EBV episomal replication capacity. EBV episomes have been reported to range up to 90 copies per cell (15). Although both inducible promoters studied here (*GRP78* and $hMTII_A$ genes) were inducible when episome-based and expressed in T cells, the fact that they demonstrated significant basal activity in the absence of their respective inducers could limit their usefulness for expression studies.

Using CD8 as a model T-cell-associated molecule, we have further demonstrated that episome-based, RSV 3' LTR-driven expression systems can be used for anti-sense RNA-mediated gene inhibition. This approach can now be extended to anti-sense RNA work in other human hematopoietic and nonhematopoietic cell types. The gene inhibition achieved here was effective (>95% inhibition), selective (vis à vis CD3 and CD2), and reversible (by removal of the selective agent). Our results further indicated that the transfection/selection scheme did not interfere with cellular functions such as proliferation. Notably, we found no effect of inhibition of CD8, a marker for class I major histocompatibility complex-restricted cytotoxic T cells, on nonspecific cytotoxicity in our system. The possibility that the small amount of residual surface CD8 (<5%) in the α-CD8/REP1 transfectant was exerting a significant functional effect seems unlikely but cannot be definitively excluded. Since the antigenic specificity of this clone is unknown, studies of the effect of CD8 inhibition on antigen-mediated recognition await stable transfection of α-CD8/REP1 into a human T-cell clone with known antigenic specificity and major histocompatibility complex restriction.

In summary, these results establish the feasibility of deriving stable functionally intact anti-sense mutants of nontransformed human T-cell clones. Furthermore, this study demonstrates the utility of episomal expression vectors for achieving efficient anti-sense RNA-mediated gene inhibition in eukaryotic cells. An episome-based system not only offers an expeditious means for achieving amplification of transfected genes, but also permits the *in vitro* manipulation of transfected gene copy number by altering the selective pressure on the episomes. The selectivity of gene inhibition, with the possibility of confounding experimental artifacts minimized, is a distinct advantage of such an anti-sense RNA-mediated mutagenesis approach. In addition, successful transfection of human T-cell clones allows for the initiation of studies of functions that are associated with nontransformed cells such as regulated growth and antigen-specific cytotoxicity. The linking of gene-directed mutagenesis and T-cell cloning technologies should now permit a more precise definition of the functional roles of a variety of specific molecules in human T lymphocytes.

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Antisense nonmuscle myosin heavy chain and c-myb oligonucleotides suppress smooth muscle cell proliferation in vitro.

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Smooth muscle cell (SMC) proliferation is a poorly understood process that plays a critical role in several pathological states, including atherosclerosis and hypertension. Recent work suggests that the oncogene c-myb and myosin, a ubiquitous cytoskeletal protein, may be directly involved in this process. We have used antisense nonmuscle myosin heavy chain (NMMHC) or c-myb phosphorothiolate oligonucleotides to inhibit proliferation of SMCs in vitro. The suppression of growth is accompanied by reductions in the concentrations of NMMHC and c-myb mRNAs as well as decreases in the levels of the corresponding proteins. The specificity of the antiproliferative effect is underscored by the absence of any detectable growth inhibition with sense NMMHC or c-myb phosphorothiolate oligonucleotides, an antisense c-myb mismatch phosphorothiolate oligonucleotide, or an antisense thrombomodulin phosphorothiolate oligonucleotide. Furthermore, the treatment of SMCs with antisense phosphorothiolate oligonucleotides for as little as 2 hours causes maximal inhibition of cell growth over the next 72 hours. Under these conditions, SMCs attain normal rates of growth over the following 48 hours, which shows that proliferation is suppressed in a reversible fashion by antisense phosphorothiolate oligonucleotides. These experiments indicate that both c-myb and nonmuscle myosin play critical roles in SMC proliferation and that reductions of either mRNA by antisense phosphorothiolate oligonucleotides arrest the process.

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1: Cancer Res. 1991 Aug 1;51(15):3996-4000.

Inhibition of c-myc expression by phosphorothioate antisense oligonucleotide identifies a critical role for c-myc in the growth of human breast cancer.

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In search of critical genes in the mechanism of estrogen action in human breast cancer, we previously showed that estrogen stimulates transcription of the c-myc gene in estrogen-dependent (MCF-7) cells. We have now examined the role of c-myc in estrogen-stimulated growth of MCF-7 cells through the use of a synthetic antisense c-myc phosphorothioate oligonucleotide to specifically inhibit expression of the c-myc protein. Estrogen induces a 5-fold increase in c-myc protein expression within 90 min in steroid-deprived cells, as detected by Western blot. Prior exposure of MCF-7 cells to 10 microM c-myc antisense oligonucleotide results in up to 95% inhibition of the c-myc protein expression induced by estrogen. Antisense-myc oligonucleotide inhibits estrogen-stimulated cell growth by up to 75% over 9 days and also exerts a cytostatic effect on the growth of estrogen-independent MDA-MB-231 cells which show relatively high, constitutive expression of c-myc. Sense-myc and antisense-pS2 oligonucleotides have no effect on c-myc protein level or growth in either cell line. These results demonstrate both the specific and durable effects of antisense phosphorothioate oligonucleotides. Furthermore, these results indicate a critical role for c-myc in the growth of breast cancer cells and support the hypothesis that loss of estrogen regulation of this gene may be an important factor in the progression of breast cancer.

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1: Eur J Biochem. 1989 Sep 1;184(1):39-45.

Antisense oligonucleotide inhibition of encephalomyocarditis virus RNA translation.

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We report the inhibition of encephalomyocarditis virus (EMCV) RNA translation in cell-free rabbit reticulocyte lysates by antisense oligonucleotides (13-17-base oligomers) complementary to (a) the viral 5' non-translated region, (b) the AUG start codon and (c) the coding sequence. Our results demonstrate that the extent of translation inhibition is dependent on the region where the complementary oligonucleotides bind. Non-complementary and 3'-non-translated-region-specific oligonucleotides had no effect on translation. A significant degree of translation inhibition was obtained with oligonucleotides complementary to the viral 5' non-translated region and AUG initiation codon. Digestion of the oligonucleotide:RNA hybrid by RNase H did not significantly increase translation inhibition in the case of 5'-non-translated-region-specific and initiator-AUG-specific oligonucleotides; in contrast, RNase H digestion was necessary for inhibition by the coding-region-specific oligonucleotide. We propose that (a) 5'-non-translated-region-specific oligonucleotides inhibit translation by affecting the 40S ribosome binding and/or passage to the AUG start codon, (b) AUG-specific oligonucleotides inhibit translation initiation by inhibiting the formation of an active 80S ribosome and (c) the coding-region-specific oligonucleotide does not prevent protein synthesis because the translating 80S ribosome can dislodge the oligonucleotide from the EMCV RNA template.

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1: J Immunol. 1988 Apr 1;140(7):2431-5.

Specific inhibition of c-myc protein biosynthesis using an antisense synthetic deoxy-oligonucleotide in human T lymphocytes.

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C-myc protein expression in human T cells was specifically inhibited by a 15-mer deoxy-oligonucleotide complementary to the 5' end of the human c-myc gene second exon. The oligonucleotide penetrates the cells without any treatment, with a plateau of cell association reached in 2 h. The oligonucleotide specifically blocked the de novo synthesis of c-myc protein, induced by PHA in human resting peripheral T cells, without impairing the overall synthesis of other proteins, as shown by two-dimensional analysis of [35S]methionine pulse-labeled proteins. The specific inhibition of c-myc protein synthesis prevented the entry into S phase of resting T cells, induced to proliferate by PHA, or IL-2-dependent T cells induced by IL-2, as shown by [3H]thymidine incorporation. The inhibition of proliferation was specific since it was not observed with the corresponding sense-oligonucleotide and was reversed by preincubation of the cells with an excess of sense oligonucleotide. These results clearly support a role for c-myc protein in the proliferation process and show that inducible protein expression can be blocked by means of synthetic oligonucleotides complementary to a coding exon.

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Suppression of Basic Fibroblast Growth Factor Expression by Antisense Oligodeoxynucleotides Inhibits the Growth of Transformed Human Astrocytes*

(Received for publication, July 27, 1990)

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Basic fibroblast growth factor (bFGF) is a heparin-binding protein expressing potent mitogenic and angiogenic properties. Elevated levels of bFGF have recently been described in human glioma cell lines. The high degree of vascularity and invasiveness which characterize human gliomas suggest that activated expression of bFGF or similar proteins may be related to the aberrant growth patterns of these tumors. The influence of endogenous bFGF on glioma cell growth *in vitro* was evaluated in the present study by down-regulating bFGF expression using antisense oligonucleotide primers. The addition of 50 μM bFGF-specific antisense primer to the human glioma cell line SNB-19 resulted in an 80% inhibition in glioma growth. This effect was saturable and specific. Antisense primers directed to two different sites of bFGF mRNA were effective in suppressing SNB-19 growth, whereas sense strand primer was ineffective. Furthermore, only the antisense primer significantly reduced the specific activity of bFGF protein in SNB-19 cell extracts. Neither antisense or sense primers inhibited the growth of non-transformed human glia. bFGF mRNA was detected in both transformed and non-transformed human glia by polymerase chain reaction analysis suggesting that alterations in bFGF isoform content or activity may be specifically related to abnormal growth control in human gliomas.

Basic fibroblast growth factor (bFGF)[1] is a multifunctional protein recognized primarily for its mitogenic and angiogenic properties. On the basis of cell culture studies, bFGF has been shown to be mitogenic for a wide range of cell types derived from mesoderm and neuroectoderm. In addition to the many *in vitro* studies performed with bFGF, it is also active in numerous *in vivo* models of angiogenesis and wound healing (1, 2). bFGF has been identified in many normal and malignant tissues (3, 4), and at several developmental time points (3, 5, 6), implying that it may play a role in normal tissue function, embryonic development, and neoplastic progression.

The mammalian central nervous system is a particularly abundant source of bFGF. Substantial quantities of bFGF have been purified from whole brain extracts, hypothalamus, and retina (7-9). Despite its abundance in neural tissue, a precise cellular localization for bFGF synthesis in brain has not been unequivocally determined. bFGF immunoreactivity has been localized to neurons *in vitro* (10) and *in vivo* (11, 12) by immunocytochemical analysis. In a more recent survey enhanced bFGF immunoreactivity was observed in brain regions enriched in neurons (13). In contrast, bFGF has also been identified in cultured mouse cerebellar astroglia (14) and in reactive rat astrocytes surrounding a focal suction wound to the brain (15). These data imply that astrocytes may represent a potential source of bFGF expression in the central nervous system under appropriate circumstances.

This hypothesis is consistent with the recent identification of bFGF in human glial tumors and in transformed human glial cell lines (16, 17). In addition to expressing bFGF, human glioma cells respond to it with increased proliferation (17, 18), suggesting that bFGF may be involved in an autocrine pathway regulating glioma growth and invasion. Due to its multifunctional properties bFGF could potentially influence glioma development by directly stimulating tumor cell growth or by promoting tumor vascularization. bFGF and related members of the FGF family have been implicated in the autocrine regulation of human tumor growth based partly on transfection studies with bFGF expression vectors, which result in amplified autocrine growth in monolayer culture and soft agar (19-21). In this report we provide evidence that bFGF expression can be altered in human glioma cells following application of bFGF-specific antisense oligonucleotides. The results of these studies indicate that bFGF expression occurs in both non-transformed and transformed human glial cells. However, only the growth of transformed human glial cells is suppressed in the presence of bFGF-specific antisense primers. Thus, while bFGF may normally be expressed by astrocytes, elevated levels or aberrant forms of bFGF may predispose astrocytes to uncontrolled cell growth.

MATERIALS AND METHODS

Materials were obtained from the following sources. Ham's F-12/Dulbecco's modified Eagle's medium (1:1) was from Irvine Scientific; fetal calf serum was from GIBCO; selenium, transferrin, hydrocortisone, and insulin were from Sigma; CHAPS detergent, protease inhibitors, anti-GFAP and anti-fibronectin antibodies were from Boehringer Mannheim; alkaline-phosphatase conjugated goat anti-mouse antibody was from Promega; purified human recombinant-bFGF was generously provided by Synergen, Inc. (Boulder, CO); the mouse monoclonal anti-bFGF antibody was generously provided by Drs. Janet L. Gross and Thomas Reilly of E. I. Du Pont de Nemours and Co., Inc.; the SNB-19 human glioma cell line was the generous gift of Dr. Paul Kornblith at Montefiore Medical Center (Bronx,

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[1] The abbreviations used are: bFGF, basic fibroblast growth factor; PDGF, platelet-derived growth factor; AS, antisense; SFM, serum-free medium; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; GFAP, glial fibrillary acid protein; PBS, phosphate-buffered saline.

NY). The RPMI-7272 human melanoma line was provided by Dr. Janet L. Gross.

Cell Culture—The SNB-19 cell line was derived from a high grade glioblastoma. The derivation of this tumor was confirmed by histological analysis as described previously (22). The SNB-19 glioma cell line expressed the astrocyte antigen, glial fibrillary acidic protein (GFAP), confirming its glial origin. The glioma cell line was maintained as described previously and was mycoplasma free (22).

Non-transformed human astrocytes were derived from surgically resected brain biopsies (temporal lobe). Astrocyte cultures were established from explants from which the meninges was surgically removed. Astrocytes were identified based on their positive expression of GFAP and their lack of staining for fibronectin as previously described (23). Astrocyte cultures were maintained in the same basic nutrient mixture as the glioma cells. The cells were shifted to a chemically defined medium for all cell growth studies involving sense and antisense primers, which consisted of Ham's F-12/Dulbecco's modified Eagle's medium (1:1) (serum-free, SFM) supplemented with prostaglandin F2α (500 ng/ml), transferrin (50 μg/ml), 50 nM hydrocortisone, putrescine (100 μM), and insulin (5 μg/ml).

Cell Growth and Dose Response—Glioma cells and non-transformed astrocytes were plated at 1×10^4 cells/2.1 cm^2 tissue culture well in serum-supplemented medium (10%). Within 18–20 h postplating, the serum-supplemented medium was removed, and the cells were washed three times with phosphate-buffered saline (PBS) and converted to SFM or chemically defined medium. Sense or antisense primers were solubilized in sterile PBS and added directly to the cells at the time of conversion to SFM or chemically defined medium. This was considered as day 0. Cell cultures were in the growth phase at the time of primer addition. 5–8 days later the cells were washed twice with PBS and removed from the tissue culture wells by trypsinization (0.25% in PBS). Cell number was determined using a hemocytometer.

Preparation of Cell Extracts—Tissue culture cells were grown to confluence in 60-mm (21 cm^2) dishes. Cells were washed with PBS, trypsinized, and pelleted. The pellet was washed twice with ice-cold PBS, resuspended, and sonicated in 10 mM Tris-HCl, pH 7.0, 2 M NaCl, 0.1% CHAPS detergent, 10 μg/ml leupeptin, 0.2 mM phenylmethylsulfonyl fluoride, and 1 μg/ml of the following protease inhibitors: antipain, elastinol, bestatin, pepstatin, 4-amidinophenylmethanesulfonyl fluoride. Sonication was performed on ice. The sonicate was incubated with 1 unit/μl of DNase for 10 min at 4 °C. The resulting supernatant was centrifuged at 14,000 × *g* for 15 min to pellet membranes. The supernatant was removed and stored at −70 °C. Aliquots were taken for protein determination by the method of Lowry (24).

bFGF was quantitated by applying aliquots of each extract to nitrocellulose sandwiched in a slot-blot apparatus. Each aliquot was added in a total volume of 50 μl in PBS. Following the addition of extract, each well of the slot blot was washed twice with PBS. After removing the nitrocellulose membrane from the slot-blot apparatus, residual protein-binding sites on the nitrocellulose were blocked by incubating the paper in blocking buffer (10 mM Tris-HCl, 150 mM NaCl, pH 8.0, 0.05% Tween 20, 1% bovine serum albumin) for 30 min at room temperature. The paper was incubated with a mouse monoclonal anti-bFGF antibody for 1 h at room temperature as previously described (17). After extensive washing with Tris-buffered saline, the sheets were incubated for 1 h at room temperature with an alkaline-phosphatase-conjugated goat anti-mouse antibody. The sheets were extensively washed in Tris-buffered saline and developed in 100 mM Tris-HCl, 100 mM NaCl, 5 mM $MgCl_2$, pH 9.5, containing the substrates nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate. Color development proceeded for approximately 4–7 min. The reaction was stopped following several rinses in deionized water.

The density of each immunoreactive band was measured using a scanning densitometer and compared with a human recombinant bFGF standard curve. The human recombinant bFGF standard curve was linear between the range of 1–250 ng of bFGF. Detection of bFGF in aliquots of extracts ranging between 0.5 and 5 μl also gave linear responses on the slot-blot and normally fell within the linear range of the bFGF standard curve. Only values falling within the linear range of the bFGF standard curve were used to quantitate the bFGF content of cellular extracts.

DNA Amplification—The presence of bFGF and bFGF receptor mRNA was demonstrated by amplifying respective target sequences using the polymerase chain reaction technique. Total RNA (1 μg) from human glioma cell lines, non-transformed human astrocytes, normal human skin keratinocytes (generously supplied by Dr. Gary Shipley, Oregon Health Sciences University), and a human melanoma cell line was used to prepare cDNA primed with random primers according to the specifications recommended by the manufacturer of reverse transcriptase H (Bethesda Research Laboratories). Polymerase chain reaction was performed on the entire cDNA product by using Thermus aquaticus (Taq) DNA polymerase (Perkin Elmer-Cetus Instruments) with the manufacturer's recommended buffers. 25 nucleotide primers beginning with codon 20 at the 5′ end of the human bFGF mRNA (25) (5′-GCC-TTC-CCG-CCC-GGC-CAC-TTC-AAG-G-3′) and complementary to codons 71–79 at the 3′ end of the bFGF mRNA (5′-GCA-CAC-ACT-CCT-TTG-ATA-GAC-ACA-A-3′) were synthesized by Research Genetics (Huntsville, AL). The predicted amplification product for human bFGF was 179 nucleotides. 22 nucleotide primers corresponding to nucelotides 955–976 at the 5′ end (5′-GAC-GCA-ACA-GAG-AAA-GAC-TTG-T-3′) and complementary to nucelotides 1594–1615 (5′-GCC-AGC-AGT-CCC-GCA-TCA-TCA-T-3′) at the 3′ end of the mRNA for the bFGF receptor (26, 27) were synthesized by Research Genetics (Huntsville, AL). The predicted amplification product for human bFGF receptor was 617 nucleotides.

Reaction conditions for reverse transcription were as follows: 1 mM each dNTP, 1 unit of RNasin, 100 pmol of random hexamer, 1 μg of total RNA, and 200 units of MoMuLV reverse transcriptase H. The reaction was run at 37 °C for 1 h. The reaction mixtures were then heated at 95 °C for 5 min to denature the RNA-cDNA hybrids and quick-chilled on ice.

The amplification reaction mixture consisted of adding 50 pmol each of upstream and downstream primers and 1 unit of Taq DNA polymerase to the reverse transcription reaction. The reaction mixture was layered with 100 μl of mineral oil to prevent evaporation. Conditions for amplification were as follows: 30 cycles at 97 °C × 1 min (denaturation), 63 °C × 1 min (primer annealing), and 72 °C × 3 min (primer extension). Amplification products were visualized on a 1.5% agarose gel developed in 1 × Tris acetate/borate buffer. The gel was stained with ethidium bromide and viewed on a UV light box.

RESULTS

Oligonucleotide primers (15-mers) corresponding to different sites of the sense or antisense bFGF mRNA were synthesized in an unmodified form (Table I). These primers were directed against either the translation initiation site (AUG codon, referred to as AS-1), or codon 60, the first splice donor-acceptor site (referred to as AS-2) (25). Antisense primer corresponding to the initiation site of the B-chain of human platelet-derived growth factor (PDGF) (28) was also synthesized. Despite extensive sequence similarity between bFGF and other members of the FGF family the sequences of the primers were sufficiently divergent from the other related gene sequences to prevent inappropriate duplex formation (29).

SNB-19 human glioma cells maintained a linear rate of growth after conversion from SSM to SFM (Fig. 1). They exhibited approximately three cell doublings over an 8-day period when maintained in SFM alone. In marked contrast, SNB-19 cell growth was dramatically suppressed following the addition of bFGF-specific antisense primer AS-2 (25 μM). AS-2 inhibited SNB-19 cell growth by 51% compared with a SFM control. This effect appeared to be specific since an equivalent concentration of the corresponding sense strand primer, S-2, had no influence on cell growth. The growth inhibitory actions of primer AS-2 did not result from primer toxicity since SNB-19 cells resumed a normal growth rate after the medium containing primer AS-2 was washed out and replaced by SFM alone. Furthermore, growth inhibition resulting from primer AS-2 could be overridden by adding exogenous bFGF to the medium (Fig. 2). The addition of bFGF (10 ng/ml) to AS-2 treated SNB-19 cells resulted in the same cell density as that observed in SFM alone or S-2-treated cultures. These results suggest that AS-2 treatment does not render SNB-19 cells metabolically or bFGF incompetent.

AS-2 induced growth inhibition was dose-dependent and

TABLE I

Location and structure of oligonucleotide primers

Two introns interrupt the human bFGF coding sequence (25). The first intron interrupts the coding sequence at codon 60. Sense and antisense primers corresponded to codons 58–62 which span the first splice-donor acceptor site. The start site refers to the translation initiation (ATG) site. h-bFGF, human basic fibroblast growth factor; h-PDGF-B, human platelet-derived growth factor-B-chain.

Growth factor	Primer	Location	Sequence
h-bFGF	Sense	Codon 58	5′-CCT-CAC-ATC-AAG-CTA-3′
h-bFGF	Antisense	Codon 58	5′-TAG-CTT-GAT-GTG-AGG-3′
h-bFGF	Antisense	Start site	5′-GGC-TGC-CAT-GGT-CCC-3′
h-PDGF-B	Antisense	Start site	5′-GCG-ATT-CAT-GCC-GAC-3′

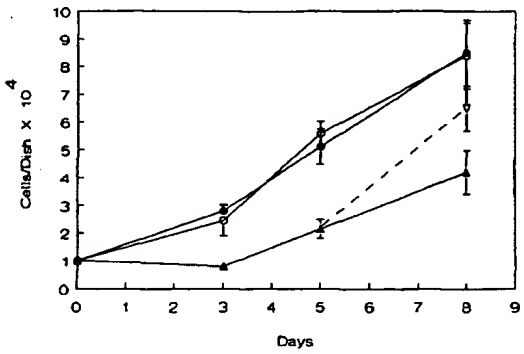

FIG. 1. **Inhibition of glioma cell growth in the presence of bFGF-specific antisense oligonucleotide primers.** SNB-19 cells were plated at a density of 1×10^4 cells/2.1 cm^2 well in serum-supplemented medium (10% FCS). 18 h later the cells were washed three times with PBS and converted to SFM alone (●), SFM plus 25 μM sense primer (○), or SFM plus 25 μM antisense primer AS-2 (▲). One set of wells was maintained in medium with antisense primer AS-2 (25 μM) for 5 days, washed three times with PBS, and converted back to SFM alone (▽). At the appropriate time points the cells were washed two times with PBS, trypsinized, and counted using a hemocytometer. The data represents the number of cells/dish ± S.D. Duplicate wells were used for each time point, and the results represent the average of two separate experiments.

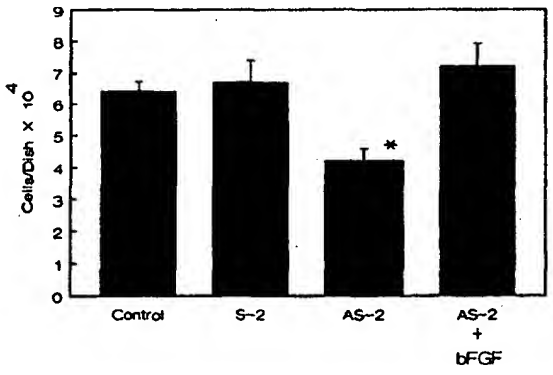

FIG. 2. **Exogenous human recombinant-bFGF restores SNB-19 cell growth in the presence of bFGF-specific antisense primer.** SNB-19 cells were plated and maintained as described in the legend to Fig. 1. 18 h after plating, the cells were washed three times with PBS and converted to SFM alone, SFM plus bFGF-specific sense primer S-2 (25 μM), SFM plus bFGF-specific antisense primer AS-2 (25 μM), or SFM plus bFGF-specific antisense primer AS-2 (25 μM) and human recombinant bFGF (10 ng/ml). 8 days after conversion to SFM plus experimental variables, cells were trypsinized and counted using a hemocytometer. Two wells were used per condition, and the data represent the average of two separate experiments ± S.D. * Differs from all other conditions at $p < 0.01$.

saturable (Fig. 3). The observed saturability suggests that the inhibitory actions of the primer were specific and presumably resulted from limiting concentrations of intracellular bFGF.

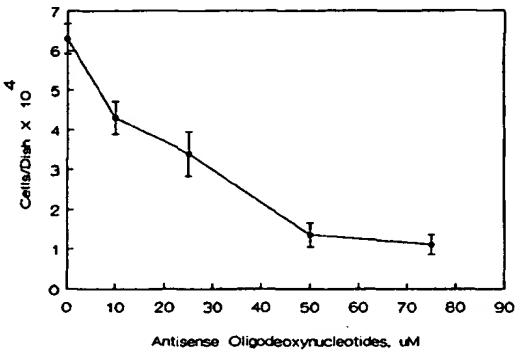

FIG. 3. **Growth response of SNB-19 cells as a function of bFGF-specific antisense oligonucleotide primer (*AS-2*) concentration.** SNB-19 cells were plated and maintained as described in the legend to Fig. 1. 18 h after plating, the cells were washed three times with PBS and converted to SFM with varying concentrations of bFGF-specific antisense primer AS-2. 8 days after conversion to SFM plus the antisense primer, cells were trypsinized and counted using a hemocytometer. Two wells were used per concentration, and the data represent the average of two separate experiments ± S.D.

AS-2 had a demonstrated K_i of 22 μM. Growth inhibition was optimal when AS-2 was added in the 50–75 μM range resulting in a 75% reduction in cell growth. In contrast, sense primer, S-2, did not inhibit SNB-19 cell growth when tested at concentrations up to 35 μM. When tested at concentrations between 50–75 μM S-2 nonspecifically reduced cell growth by 10–15%.

The specificity of antisense primer AS-2 was evaluated by testing a second antisense primer complementary to a different site on the bFGF mRNA. A second antisense primer was synthesized corresponding to the initiation translation site of bFGF (AS-1, Table I). Primer AS-1 suppressed SNB-19 cell growth just as effectively as primer AS-2 suggesting that growth inhibition was due to the formation of specific complexes between antisense primers and endogenous bFGF mRNA (Fig. 4). Primer AS-1 also showed the same dose-dependent influence on growth inhibition as primer AS-2 (data not shown). An antisense primer unrelated to bFGF was also evaluated. This corresponded to the initiation translation site of the B-chain of human PDGF, a growth factor previously demonstrated in human gliomas (30) (Table I). PDGF has been implicated as a potential autocrine regulator of glioma growth (31). Surprisingly, the antisense primer directed against the B-chain of human PDGF did not effect SNB-19 cell growth when tested at concentrations equivalent to those used for bFGF (Fig. 3). Thus, the growth inhibitory actions of the bFGF antisense primers appear to be specific and due to the formation of specific hybrids between the antisense primers and their respective mRNA.

The lack of growth inhibition observed with bFGF-specific sense primer S-2 and the PDGF-B-chain-specific antisense primer suggested that the inhibitory effect of the bFGF-

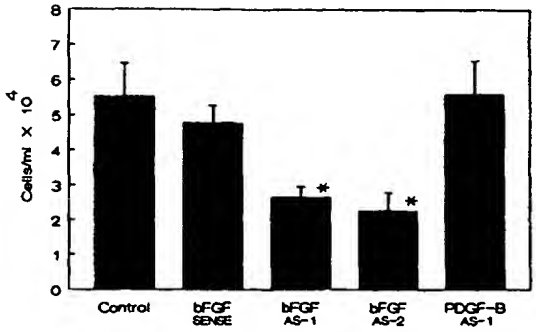

FIG. 4. **Growth response of SNB-19 cells to bFGF-specific and unrelated antisense oligonucleotide primers.** SNB-19 cells were plated and maintained as described in the legend to Fig. 1. 18 h after plating, the cells were washed three times with PBS and converted to SFM alone (*control*), SFM plus bFGF-specific sense primer (*S-2*, 35 μM), SFM plus bFGF-specific antisense primer AS-1 (35 μM), SFM plus bFGF-specific antisense primer AS-2 (35 μM), and SFM plus PDGF B-chain-specific antisense primer AS-1 (35 μM). 8 days after conversion to SFM plus the respective oligonucleotide primer, cells were trypsinized and counted using a hemocytometer. Two wells were used per condition, and the data represent the average of three separate experiments ± S.D. * Differs from control, bFGF sense, and PDGF-B AS-1 at $p < 0.01$.

specific antisense primers was related to specific alterations in bFGF expression. We investigated this possibility by measuring bFGF protein in SNB-19 cells using a slot-blot immunodetection technique. bFGF protein was quantitated by slot-blot analysis against a standard human recombinant bFGF curve. The monoclonal antibody used in the detection of bFGF was previously shown to be specific for human bFGF and did not cross-react with human acidic fibroblast growth factor (17). Furthermore, the antibody recognized the appropriate molecular weight forms of bFGF in SNB-19 cells as judged by Western blot analysis (17).[2] SNB-19 cells grown in serum-free medium until approximately 75% confluent exhibited 5.56 ng of bFGF/μg of protein (Table II). The sense strand primer, which did not effect SNB-19 cell growth, correspondingly had no effect on SNB-19 bFGF content. In marked contrast, antisense primer AS-1 (35 μM) significantly reduced bFGF expression in SNB-19 cells. The 67% reduction in bFGF content was paralleled by a 55% reduction in cell number (data not shown), implying that inhibition of SNB-19 cell growth was directly related to the loss of bFGF.

The relevance of bFGF expression to the growth of human glioma cells was addressed further by investigating the actions of bFGF antisense primers on the growth of non-transformed human astrocytes. Non-transformed human astrocytes were prepared from surgically resected temporal lobe biopsies. Cells prepared in this manner were identified as astrocytes based upon their expression of the astrocyte intermediate filament protein, GFAP (data not shown). As shown in Table III, bFGF-specific sense and antisense primers failed to alter the growth of non-transformed human astrocytes. Nevertheless, the same concentration of antisense primers (25 μM) resulted in a 51% inhibition in SNB-19 cell growth. The observation that bFGF antisense primers do not effect the growth of non-transformed human astrocytes suggests that bFGF is not normally expressed in these cells but is activated as a result of neoplastic transformation.

In order to determine if the absence of bFGF in non-transformed human astrocytes accounted for the lack of growth inhibition by bFGF-specific antisense primers, we studied the expression of bFGF mRNA in these cells. This

[2] J. Gross, manuscript in preparation.

TABLE II

Influence of bFGF sense and antisense oligonucleotides on the bFGF content of SNB-19 cells

SNB-19 cells were plated at a density of 5×10^5 cells/21-cm^2 plate in serum-supplemented medium (10% fetal calf serum). 18 h later the cells were washed three times with PBS and converted to SFM alone (control), SFM plus bFGF-specific sense primer AS-2 (35 μM) or SFM plus bFGF-specific antisense primer AS-1 (35 μM). 4 days after conversion to SFM plus primers the cells were trypsinized and counted. Equal numbers of cells were removed from each treatment (3×10^6/plate), and cell extracts were prepared as described under "Materials and Methods." Extracts were evaluated for protein content and administered to nitrocellulose using a slot-blot apparatus. bFGF immunoreactive bands were visualized using alkaline phosphatase-conjugated secondary antibodies as described under "Materials and Methods." The nitrocellulose was washed extensively with water to stop the color reaction, dried, and scanned using a densitometer. The bFGF content of the various extracts was determined by comparison with a human-recombinant bFGF standard curve. The data are expressed as the content of bFGF/μg of extract protein. All immunoreactive bands fell within the linear range of the human recombinant-bFGF standard curve. Different amounts of extract were also evaluated to confirm that the amount of extract added to the slot-blot was not saturating. The extract from a single plate was tested in duplicate. The data represent the average of four separate determinations ± S.D. * Differs from control and sense primer at $p < 0.01$.

Condition	Conc.	bFGF content[a]	% Reduction
	μM		
Control		5.71 ± 0.37	
Sense primer	35	6.44 ± 0.12	0
Antisense primer	35	1.87 ± 0.31*	67.25

[a] ng/μg protein.

TABLE III

Influence of various growth factor sense and antisense primers on the growth of non-transformed human astrocytes in culture

Non-transformed human astrocytes were prepared from temporal lobe biopsies as described under "Materials and Methods." These cells were plated at a density of 1.5×10^4 cells/2.1 cm^2 well in serum-supplemented medium (10%). 18 h later the cells were washed three times with PBS and converted to chemically defined medium (CDM) alone, CDM plus bFGF-specific sense primer S2, CDM plus bFGF-specific antisense primer AS-1, or CDM plus bFGF-specific antisense primer AS-2. 8 days after conversion to CDM or CDM plus the appropriate primers, cells were trypsinized and counted. The data is expressed as the average number of cells/well ± S.E. 2 wells were used per condition, and the data represent the average of two separate experiments.

Condition	Conc.	Cells/well × 10^4 ± S.D.
	μM	
CDM		4.00 ± 0.20
CDM + bFGF (S-2)	25	3.80 ± 0.00
CDM + bFGF (AS-2)	25	3.60 ± 0.60
CDM + bFGF (AS-1)	25	3.89 ± 0.08
CDM + PDGF-B (AS-1)	25	3.80 ± 0.57

was accomplished by amplifying bFGF mRNA target sequences using polymerase chain reaction. Amplification of mRNA obtained from two different malignant human glioma cell lines yielded the expected amplification product of 179 base pairs (Fig. 5*A*, *lanes 2* and *3*). bFGF mRNA was also detected in non-transformed human astrocytes (*lane 4*). A human melanoma cell line also expressed bFGF mRNA (*lane 5*), although at reduced levels compared with transformed and non-transformed astrocytes. This same line expressed approximately 20-fold less bFGF protein by slot-blot analysis.[2] Normal human skin keratinocytes were used as a negative control, since these cells have been shown to express low to undetectable levels of bFGF mRNA by Northern blot analysis (32, 33). As seen in *lane 6* (Fig. 5), normal human skin

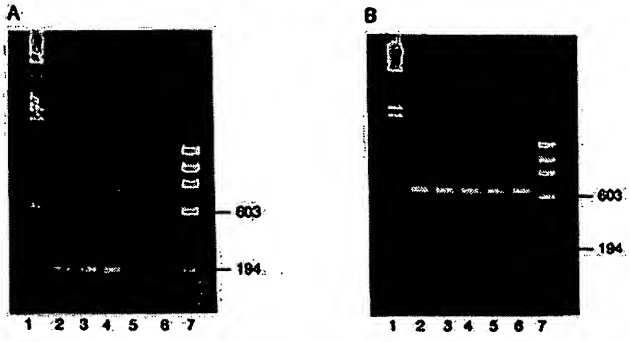

FIG. 5. Analysis of bFGF and bFGF-receptor mRNA by polymerase chain reaction. 1 μg of total RNA from SNB-19 glioma cells (*lane 2*), SNB-75 glioma cells (*lane 3*), normal human astrocytes (*lane 4*), human melanoma cells (*lane 5*) and 50 ng of poly(A)$^+$ RNA from normal human skin keratinocytes (*lane 6*) was reverse transcribed and amplified for either bFGF (*A*) or bFGF-receptor (*B*) using primers described under "Materials and Methods." Following 30 cycles of amplification, 10% of the reaction mixture was resolved on a 1.5% agarose gel and visualized with ethidium bromide. *Lane 1* represents λDNA/*Hind* III size markers and *lane 7* represents φX174 RF DNA/*Hae*III fragments. bFGF primers amplified a 179-base pair fragment (*A*) and bFGF-receptor primers amplified a 617-base pair fragment (*B*) as predicted. These gels are representative of four separate experiments.

keratinocytes also expressed significantly less bFGF mRNA than brain-derived cells.

However, normal human skin keratinocytes and human melanoma cells expressed a significant amplification product for bFGF-receptor mRNA, indicating that the low abundance of amplification product for bFGF was not related to the integrity of cellular mRNA. The presence of bFGF-receptor mRNA is consistent with reports demonstrating that bFGF is a potent mitogen for both melanoma cells and human skin keratinocytes (32, 34). Transformed and non-transformed human astrocytes also expressed mRNA for the bFGF receptor, consistent with their demonstrated responsiveness to bFGF.

DISCUSSION

The over expression of growth factors and their receptors has been implicated in the genesis and maintenance of a variety of human neoplasms. The putative growth factor receptor c-*neu* (c-erbB-2) has been detected in a significant number of human breast carcinomas (35) while the epidermal growth factor receptor is amplified in approximately 30–40% of human gliomas (36). Amplification of bFGF-related int-2 and hst genes has also been observed in a small percentage of breast tumors (37).

Basic fibroblast growth factor, a potent mitogenic and angiogenic protein, has been implicated as a potential regulator of tumor cell growth. Transfection of fibroblasts (19) baby hamster kidney cells (20) and murine melanocytes (21) with bFGF cDNA expression vectors supports the concept that alterations in bFGF expression promote anchorage-independent growth and morphological transformation *in vitro*. Although there is no evidence that bFGF is directly related to malignant transformation *in vivo*, bFGF has been associated with autonomous cell growth in several types of human tumors, including melanoma (38, 39) bladder and kidney (40), rhabdomyosarcoma (41), and AIDS-Kaposi's sarcoma (42).

Our results suggest that bFGF may also confer autonomous cell growth on neoplastic glial cells. Human glioma cells have recently been shown to express bFGF mRNA (43),[3] bFGF protein (16, 17), and high affinity bFGF receptors (17). Thus, all of the components necessary for a bFGF autocrine pathway exist in some glioma cells. Addition of exogenous bFGF to human glioma cells in culture enhances cell proliferation (17, 18). Malignant glioma cells grow exceptionally well in serum-free medium and in soft agar (17, 22) which could reflect the expression of high levels of intracellular bFGF. A correlation between intracellular bFGF content and the degree of malignancy in gliomas might explain why highly malignant gliomas are less responsive to exogenous bFGF compared with normal astrocytes (23) and benign gliomas (18).

The hypothesis that glioma cell growth is promoted by an autocrine pathway involving bFGF was tested by selectively reducing bFGF expression in SNB-19 glioma cells using bFGF-specific antisense primers. The addition of the primers significantly attenuated SNB-19 cell growth and appeared to be specific. The specificity of the antisense primers was supported by the following observations. 1) A bFGF-specific sense strand primer lacked growth inhibitory activity; 2) growth inhibition was observed with two different bFGF-specific antisense primers corresponding to different sites of the bFGF mRNA; 3) a PDGF-B chain-specific antisense primer also lacked growth inhibitory activity; 4) growth inhibition was dose dependent and saturable; 5) bFGF-specific sense primers did not effect bFGF content in SNB-19 cells, while bFGF-specific antisense primers reduced the bFGF content of SNB-19 cells by 67%; and 6) bFGF-specific antisense primers did not inhibit the growth of non-transformed cells. While these results do not rule out a contribution by other growth factors to the growth of SNB-19 cells, they clearly demonstrate that endogenous bFGF promotes SNB-19 cell growth.

A large proportion of human glioma cell lines have also been shown to express PDGF genes (A- and B-chains) and produce PDGF-like growth factors (44). However, not all glioma cell lines producing PDGF-like growth factors express PDGF receptors (30, 45). Hermansson *et al.* (46) recently demonstrated the expression of all three genes (PDGF A, B, and receptor) in glioma cells by *in situ* hybridization. Interestingly, the A-chain mRNA was predominantly expressed in areas of tightly packed glioma cells. The PDGF A-chain does not stimulate DNA synthesis as effectively as the B-chain or the AB-heterodimer (47). A growth factor has recently been purified from glioma cells that is structurally similar to the PDGF A-chain homodimer (48). The glioma-derived growth factor possessed only limited mitogenic activity consistent with previous reports on the activity of A-chain homodimers. Thus, the absence of PDGF receptors on glioma cell lines expressing PDGF and the predominant expression of the weakly mitogenic PDGF A-chain by glioma cells makes it difficult to define a role for PDGF in the progression of human gliomas. In addition to PDGF, approximately 40% of malignant human gliomas overexpress the EGF receptor gene (36). Since this locus is not overexpressed in all gliomas and only about 50% of all gliomas exhibit gene amplification (49), changes in this particular locus may not be a mechanism for the formation of all malignant gliomas.

In contrast, the presence of bFGF has recently been demonstrated immunocytochemically in 16/16 human astrocytoma's and 11/12 glioblastoma's implicating bFGF in the progression of malignant gliomas (16). The mechanism by which bFGF sustains glioma cell growth is not understood. Basic FGF appears to lack a signal sequence (25, 50) that would direct its release along conventional secretory pathways. Although the occurrence of soluble bFGF has been described (20, 51), the concentrations are usually quite low, so that the vast majority of bFGF appears intracellularly bound. There-

[3] R. S. Morrison, manuscript in preparation.

fore, glioma growth could be regulated in an autocrine manner by intracellular bFGF as suggested for PDGF (52). However, the release of bFGF by unique secretory pathways or by leakage from dying cells has not been ruled out, consistent with recent reports demonstrating that secreted forms of bFGF are more effective at regulating phenotypic transformation (53–55). We have detected extremely low quantities of immunoreactive bFGF in medium conditioned by glioma cell lines (less than 0.1% of total cellular bFGF) (17). However, we have found that anti-bFGF monoclonal antibodies retard the growth of several human glioma cell lines (30%) after addition to the culture medium, suggesting the presence of extracellular bFGF.[2] Thus, we cannot rule out the possibility that a small percentage of SNB-19-derived bFGF is secreted or released from cells where it initiates its action extracellularly. Since bFGF-specific antisense primers would be expected to reduce total cellular synthesis of bFGF, its release by SNB-19 cells could also be effected.

bFGF has recently been shown to belong to a family of structurally related proteins (4). Several members of this family, hst/K-FGF, FGF-5, and KGF are synthesized with hydrophobic signal sequences that permit cellular secretion (56–58). It is not known if glioma cells express other members of the FGF family in addition to basic and acidic FGF (59). The expression of these two FGFs by glioma cells may indicate a breakdown in the regulation of this growth factor family in brain tumors.

The lack of growth inhibition observed with antisense primers on non-transformed human glia further suggests that bFGF expression is relevant to the growth of human gliomas. Non-transformed astrocytes express bFGF mRNA as shown by polymerase chain reaction analysis. Therefore, the mere presence of bFGF is not sufficient to promote abnormal cellular growth. One explanation is that glioma cells express elevated levels of bFGF compared with non-transformed astrocytes. Alternatively, glioma cells may express unique bFGF isoforms with enhanced mitogenic and angiogenic activity. It is also possible that non-transformed cells are not affected by bFGF-specific antisense primers because the total contribution made by bFGF to their growth is very small compared with its contribution to the growth of transformed glia. Alternatively, the expression of bFGF in non-transformed astrocytes may be so low that bFGF antisense primers do not effect a significant change in bFGF levels. It remains to be seen whether all bFGF mRNA transcripts can be inactivated using antisense primers or antisense vectors. Finally, we cannot rule out the possibility that changes in the bFGF receptor contribute to neoplastic progression in astrocytes. Alterations in receptor structure could influence receptor compartmentalization making the bFGF receptor more accessible to intracellular bFGF.

In summary, the present data indicate that human glial cells have the capacity to express bFGF and that alterations in FGF expression may play a role in the development and progression of human gliomas. bFGF-specific antisense primers have proven an effective tool to modulate bFGF expression in glioma cells and may eventually be applied to control the growth of these cells. Since bFGF expression is observed in both transformed and non-transformed astrocytes, characterizing the bFGF receptor and bFGF isoforms in these cells may shed light on the role of bFGF in neoplastic transformation.

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Repression of nicotinic acetylcholine receptor expression by antisense RNAs and an oligonucleotide

(*Xenopus* oocytes/*Torpedo*/hybrid-arrested translation/membrane currents/chloride channels)

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ABSTRACT **Four antisense RNAs, synthesized from cDNA clones coding for the four subunits of the acetylcholine receptor of *Torpedo* electroplaques, were used to study their effect on the expression of functional *Torpedo* acetylcholine receptors in *Xenopus* oocytes. All antisense RNAs inhibited the appearance of functional receptors in the oocyte's surface membrane for at least 1 week. This inhibition was specific because the antisense RNAs did not block the expression of the Cl^- channels, also encoded by *Torpedo* electroplaque mRNA. Experiments with incomplete antisense RNAs and a synthetic oligonucleotide indicate that covering the ribosome binding site or the initiation codon in the mRNA is not a necessary requirement for efficient blocking. Thus, the use of antisense RNAs combined with the *Xenopus* oocyte system provides a novel approach to screen cDNA libraries for the genes coding for multisubunit neurotransmitter receptors.**

It was recently discovered that gene expression in prokaryotes and in various cells, including *Xenopus* oocytes and mammalian cells, can be selectively inhibited by antisense RNA, that is, RNA that is complementary to a target RNA (see ref. 1 for a review). This inhibition sometimes involves a hybridization between an antisense RNA and its counterpart mRNA, which results in an inhibition of mRNA translation. Thus, antisense RNAs can be used for identifying a gene product of interest and studying its function as well as its role in early development.

To examine the applicability of antisense RNAs to the study of neurotransmitter receptors, which are key molecules in synaptic communication and also may play an important role in the formation of synaptic connections (2, 3), we have examined the effect of antisense RNAs on the functional expression of the multisubunit nicotinic acetylcholine (AcCho) receptor (AcChoR) of the electric organ of *Torpedo* in *Xenopus* oocytes.

MATERIALS AND METHODS

Plasmids. Full-length *Torpedo* AcChoR cDNA clones (4, 5) were provided by T. Claudio (Yale University; α, β, γ, and δ subunits) and S. Heinemann (Salk Institute; γ subunit). The cDNA inserts were excised from vectors and inserted into plasmids pSP64 (γ-subunit cDNA) or pGEM4 (α-, β-, and δ-subunit cDNAs). For *in vitro* transcription the resulting plasmids were linearized by digestion with *Hin*dIII (α), *Xba* I (γ and δ), or *Nae* I (β) and were used as templates.

mRNA Preparation. Total RNAs were extracted either from *Torpedo* electric organ or cat denervated muscles, and poly(A)$^+$ mRNAs were obtained by oligo(dT)-cellulose chromatography as described (6).

In Vitro **Transcription.** The bacteriophage SP6 or T7 RNA polymerases were used to synthesize antisense RNAs in the presence of the cap analog GpppG by using 10 μg of linearized DNA as template as described (7–9). After synthesis, RNase-free DNase was added to a concentration of 1 unit per μg of DNA. Following phenol/chloroform extraction, the RNA was recovered by precipitation with ethanol and finally was dissolved in distilled water for injection into the oocytes.

Translation in *Xenopus* Oocytes. *Xenopus* oocytes were injected (*ca.* 50 nl) with *Torpedo* mRNA (≈50 ng) alone or together with antisense RNA (≈10 ng) and cultured at 16°C in modified Barth's medium containing gentamicin (0.1 mg/ml) and nystatin (50 units per ml) as in ref. 6. In some experiments, the oocytes were incubated in the presence of [^{35}S]methionine (1 mCi/ml; 1 Ci = 37 GBq).

Immunoprecipitation. For identification of the AcChoR subunits, ^{35}S-labeled oocyte translation products were heated to 100°C in 1% $NaDodSO_4$/5 mM EDTA for 3 min and were diluted with 4 volumes of buffer (60 mM Tris chloride, pH 7.6/6 mM EDTA/190 mM NaCl/1.25% Triton X-100). After addition of rat antisera raised against $NaDodSO_4$-denatured AcChoR and incubation for at least 12 hr at 4°C, immunocomplexes were adsorbed to protein A-Sepharose gel. The gels were then processed as described (10) and analyzed by $NaDodSO_4$ gel electrophoresis (11).

Electrophysiology. This was carried out as described (6, 12) with the oocyte membrane potential clamped at −60 mV. Atropine (0.5 − 1.0 μM) was used to block any possible muscarinic responses to AcCho (12).

Other. Restriction enzyme digestions were carried out as instructed by the supplier. The oligonucleotide was synthesized by using an automatic DNA synthesizer (Applied Biosystems, Foster City, CA).

RESULTS

Effect of Antisense RNAs on the Expression of *Torpedo* AcCho Receptors. It is well known that the AcChoR of *Torpedo* is a heteropolymer composed of five subunits of four different types α_2, β, γ, and δ (for reviews, see refs. 13–16). To obtain sufficient quantities of the four subunit-specific antisense RNAs, the cDNAs of the AcChoR subunits were inserted into plasmid vectors containing a phage SP6 promoter or both SP6 and T7 promoters. *In vitro* transcription of linearized plasmids by either SP6 or T7 polymerases (see *Materials and Methods*) generated pure preparations of the subunit-specific antisense RNAs (Fig. 1*A*). Typically, we obtained 1–2 μg of capped antisense RNA per μg of DNA.

We have shown (11, 17, 18) that injection of *Torpedo* electroplaque mRNA into *Xenopus* oocytes leads to the synthesis of the receptor subunits and to the incorporation of

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Abbreviations: AcCho, acetylcholine; AcChoR, acetylcholine receptor.

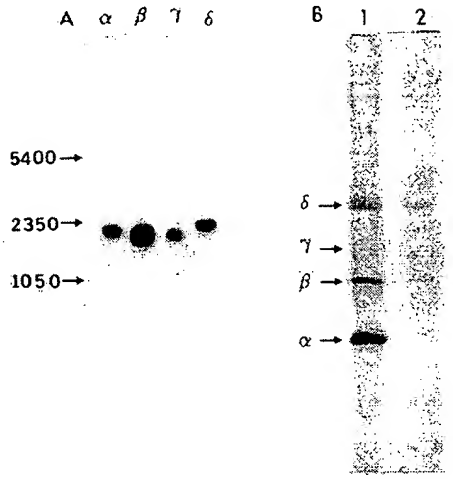

FIG. 1. (*A*) Agarose gel electrophoresis analysis of AcChoR subunit-specific antisense RNAs synthesized *in vitro*. The length standard was *Hin*dIII-digested phage PM2. (*B*) $NaDodSO_4$ gel electrophoretic analysis of the AcChoR subunits synthesized in oocytes and immunoprecipitated with antibodies raised against $NaDodSO_4$-denatured AcChoR. The immunoprecipitates were obtained from 10 oocytes injected with *Torpedo* mRNA alone (lane 1) and *Torpedo* mRNA with antisense α-subunit RNA (lane 2). The positions of the AcChoR subunits purified from *Torpedo* electric organ are marked by arrows.

functional *Torpedo* AcChoR in the oocyte's surface membrane. Therefore, we used *Xenopus* oocytes to examine the effect of each subunit-specific antisense RNA on the translation of all four subunit mRNAs and on the expression of functional AcChoRs.

To study the effect of antisense RNA on specific mRNA translation, oocytes were injected with whole *Torpedo* mRNA plus α-subunit antisense RNA and incubated in the presence of [^{35}S]methionine. The translation products were immunoprecipitated by using polyclonal antibodies against *Torpedo* AcChoR and then were separated by $NaDodSO_4$ gel electrophoresis. As a control, the *Torpedo* mRNA was translated in oocytes in the absence of antisense RNAs and processed as the test sample. The β and δ subunits were present in both samples, while the α subunit was seen only in the control sample (Fig. 1*B*). These results suggest that the α-subunit antisense RNA blocks specifically the translation of the α-subunit mRNA. In both samples, the presence of the γ subunit was not very obvious probably because of proteolysis during sample preparations, since the γ subunit is susceptible to proteolitic degradation (19). The amount of β and δ subunits in the test oocytes appeared to be less than those in the control oocytes, suggesting that in oocytes the unassembled subunits are degraded more rapidly than assembled ones, as happens in tissue-cultured muscle cells (20). However, the possibility that the α-subunit antisense RNA somehow inhibits the translation of the β- and δ-subunit mRNAs is not excluded.

To study the expression of functional AcChoRs, we measured the response to AcCho in oocytes injected with *Torpedo* mRNA alone or with one of the subunit antisense RNAs. Oocytes injected with *Torpedo* mRNA alone gave large smooth inward membrane currents in response to bath application of AcCho (Fig. 2), and the mean amplitude of the currents increased from 3,950 to 10,530 nA with longer times after mRNA injection (Table 1). In contrast, the amplitude of the currents elicited by AcCho applied to the oocytes injected with any of the antisense RNAs was greatly reduced (Fig. 2). Mean values of AcCho-activated currents at 3, 5, and 7 days after injection are shown in Table 1. At all the times examined, the strongest inhibition was consistently obtained with the α-subunit antisense RNA. In the experiment shown in Table 1, the mean currents obtained from oocytes injected with both whole *Torpedo* mRNA and the α-subunit antisense RNA were only 0.3–0.7% of that from oocytes injected with *Torpedo* mRNA alone; in other experiments, an even larger inhibition was observed. A smaller, but still large, inhibition was exerted by the β-, γ-, or δ-subunit antisense RNAs. For example, the mean amplitude of the AcCho-activated current at the various times after injection with β-subunit antisense RNA was reduced to 2.9–4.3%; with the γ- and δ-subunit antisense RNAs, it was decreased to 0.9–3.9% and 4.3–9.8%, respectively. Interestingly, with increasing time after injection, the AcCho-activated currents appeared to escape partly from the inhibition caused by the β- and δ-subunit antisense RNAs. This point will be reported in more detail at a later date.

Effect of Antisense RNA on the Expression of *Torpedo* Electroplaque Cl^- Channels. We have shown (21) that injection of *Torpedo* electric organ mRNA induces the appearance of at least two types of membrane channels in *Xenopus* oocytes: an AcCho-activated channel and a voltage-activated Cl^- channel. The Cl^- channel is activated at

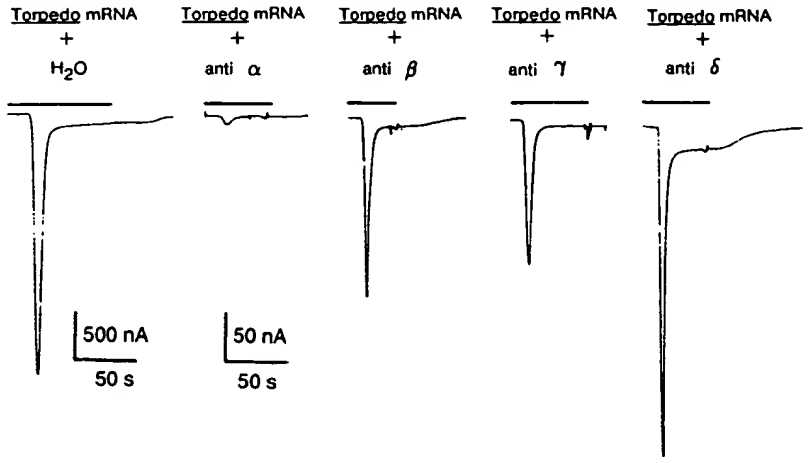

FIG. 2. AcCho-activated currents recorded in *Xenopus* oocytes injected with *Torpedo* mRNA alone or together with antisense RNAs. AcCho (100 μM) was applied by bath perfusion for the durations indicated by the bars. Atropine (0.5 μM) was used to block possible native muscarinic responses to AcCho.

Table 1. Membrane currents elicited by AcCho in *Xenopus* oocytes injected with *Torpedo* mRNA alone or together with synthetic antisense RNA

Injection*	Current elicited by 100 μM AcCho,† nA ± SEM		
	Day 3	Day 5	Day 7
Torpedo mRNA			
+ H_2O	3,950 ± 2,263 (5)	7,340 ± 1,986 (5)	10,530 ± 2,680 (4)
+ anti-α RNA	10 ± 6 (4)	20 ± 9 (5)	77 ± 55 (3)
+ anti-β RNA	115 ± 33 (4)	271 ± 56 (5)	456 ± 150 (4)
+ anti-γ RNA	152 ± 98 (4)	63 ± 22 (5)	147 ± 18 (2)
+ anti-δ RNA	170 ± 63 (4)	722 ± 237 (5)	(405) (1)

*Water was added when *Torpedo* mRNA was injected alone to make its concentration the same as when injected with antisense RNA. Anti-α (β, γ, δ) RNAs denote anti-α (β, γ, δ)-subunit antisense RNAs.

†Mean peak amplitudes of current elicited on days 3, 5, and 7 after injection. The numbers in parentheses refer to the number of oocytes. All oocytes were from the same donor.

potentials more positive than about −50 mV and is inactivated progressively at more negative potentials.

To test further whether the expression of functional AcChoRs was inhibited in a specific manner by the antisense RNAs, we also tested the oocytes for the expression of the Cl^- channels. Merely by inserting a microelectrode into the oocytes, it became evident that the antisense RNAs did not block the expression of the Cl^- channels because the resting potential was low and close to the chloride equilibrium potential, as is the case with oocytes injected with *Torpedo* electroplaque mRNA alone (21). Furthermore, the membrane conductance, measured from the current required to double the membrane potential from −20 to −40 mV, also showed that the Cl^- channel was well expressed. For example, in one experiment the membrane conductance was 18.5 ± 3.9 μS (mean ± SEM) in oocytes injected with *Torpedo* mRNA alone and 17.8 ± 3.3 μS when they were coinjected with Torpedo mRNA and α-subunit antisense RNA. Moreover, in both cases the current–voltage relation had the nonlinear behavior previously described (21). In contrast, the membrane conductance of control oocytes injected with combined synthetic α-, β-, γ-, and δ-subunit sense mRNAs was 3.1 ± 0.5 μS, and the current–voltage relation was fairly linear as in noninjected oocytes.

Length of Antisense RNAs and Potency of Repression. To examine the length of antisense RNA required for repressing the expression of functional AcChoRs, α-subunit antisense RNAs of different lengths were synthesized by using linearized DNAs at different restriction sites as illustrated in Fig. 3*A*. Each antisense RNA, having the same 5′ end but different 3′ ends, was injected into oocytes together with whole *Torpedo* electroplaque mRNA, and the oocytes were tested electrophysiologically. The antisense transcripts from the DNA linearized with *Hin*dIII or *Pvu* II abolished almost completely the expression of functional AcChoRs, and this inhibition appeared to be stable for more than 1 week (Fig. 3*B*). The *Hin*dIII antisense RNA (about 1810 bases) would cover all of the protein coding sequence and some of the 5′ and 3′ untranslated portions of the α-subunit mRNA, whereas the antisense RNA from the *Pvu* II-digested DNA template (about 1500 bases) would leave the 5′ untranslated sequence and 60 codons of protein coding sequence of the α-subunit mRNA exposed. In contrast, the antisense RNA truncated at the *Pst* I site (about 420 bases), which would cover only the 3′ untranslated region and about 1/11th of the protein coding sequence of the α-subunit mRNA, was less potent in inhibiting the expression of AcChoRs, and the inhibition was significantly reversed 9 days after injection. Thus, it is not necessary to cover the 5′ untranslated region and/or the sequence around the initiation codon to block the translation of the α-subunit mRNA; but hybridization to the 3′ untranslated sequence and/or the small portion of 3′ coding sequence is not sufficient for potent repression.

Antisense RNA Concentration and Repression Potency. To determine the amount of antisense RNA required to block the expression of functional AcChoRs, different amounts of the complete α-subunit antisense RNA were injected into the oocytes. About 10 ng of the antisense RNA per oocyte was

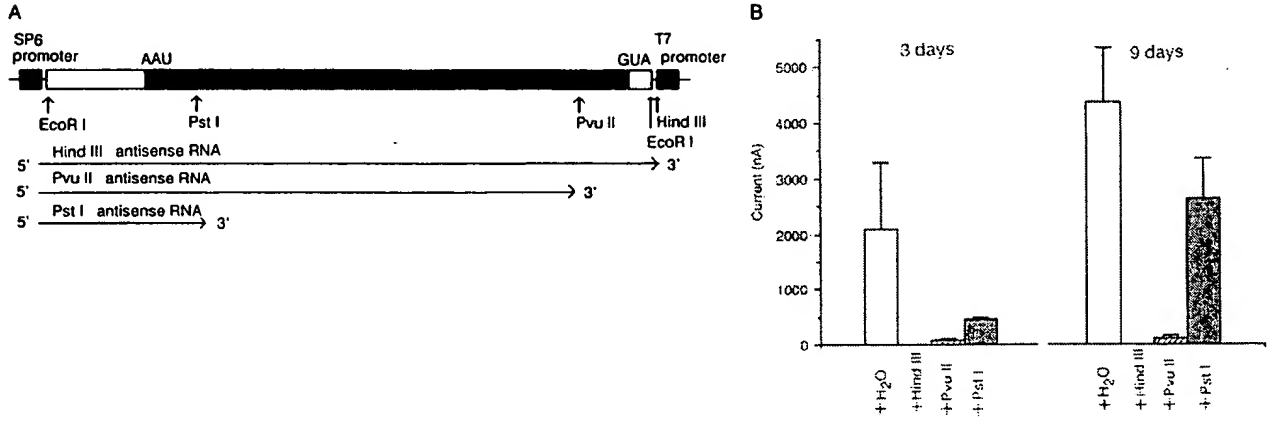

FIG. 3. (*A*) Schematic representation of linearized plasmid used as a template for synthesis of partial-length antisense RNAs. (*B*) Effect of different-length antisense α-subunit RNAs on the mean sizes of AcCho-activated membrane currents in *Xenopus* oocytes. The oocytes were injected with *Torpedo* mRNA alone or with antisense RNAs and were examined 3 or 9 days after injection. Responses were measured from records similar to those in Fig. 2. In each frame, columns (left to right) give measurements from oocytes injected with *Torpedo* mRNA alone or *Torpedo* mRNA with *Hin*dIII antisense RNA (no current), *Pvu* II antisense RNA, or *Pst* I antisense RNA. Each column represents the mean ± SEM of three to six determinations.

sufficient to block the expression of AcChoR by 99.2% in the oocytes that had been coinjected with about 50 ng of whole *Torpedo* mRNA (Fig. 4). Assuming that about 2.4% of the total mRNA in *Torpedo* electric organ is AcChoR mRNA (22), 50 ng of total mRNA would contain about 0.48 ng of the α-subunit mRNA and 0.24 ng of each of the other subunit mRNAs.

Nevertheless, 1 ng of antisense RNA was still very potent in blocking the appearance of functional AcChoRs (by >95%); even with 0.1 ng of antisense RNA, the expression was reduced to 22.1% of the control value. It should be noted that, in the last instance, the concentration of antisense RNA would be much lower than that of the target-sense mRNA, whereas repression in other systems frequently requires the antisense RNA to be in great excess. The unexpectedly large inhibition with 1 ng (and particularly with 0.1 ng) of antisense RNAs may be accounted for, at least partly, if in the oocyte an excess of the α subunit is required for the efficient assembly of functional AcChoRs, as is the case in muscle AcChoR (23).

Repression by a Synthetic Oligonucleotide. To assess further the regions of the α-subunit mRNA that can be covered for efficient inhibition of the expression of functional AcChoR, we synthesized an oligonucleotide (3′ CTTTGTGCAAACCAACGAT 5′) that is complementary to the coding sequence for amino acid residues 4–10 of the α subunit. Injection of the oligonucleotide together with whole *Torpedo* mRNA into the oocytes almost completely abolished the appearance of functional AcChoRs (Fig. 5). The mean AcCho-activated current was only 27 nA, as compared to about 2000 nA in control oocytes injected with *Torpedo* mRNA alone. Since it has been shown that injection of a large amount of DNA into *Xenopus* fertilized eggs is toxic (24, 25), it could be thought that the inhibition we observed was due to unspecific toxic effects. However, this oligonucleotide was much less effective in blocking the functional expression of cat muscle AcChoRs (about 47% inhibition) in oocytes injected with denervated cat muscle mRNA (see ref. 6). Furthermore, oocytes injected with *Torpedo* mRNA and the oligonucleotide were still able to express the Cl^- channel as efficiently as oocytes injected with *Torpedo* mRNA alone. Thus, it is very likely that the oligonucleotide inhibited the expression of the *Torpedo* AcChoR in a specific manner.

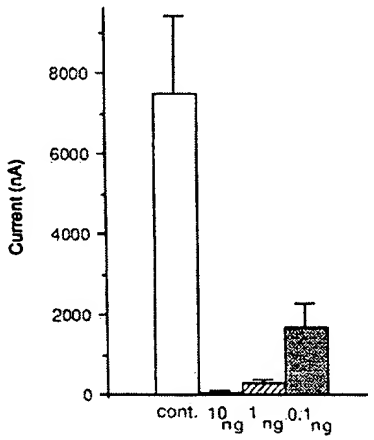

FIG. 4. Relation between the amount of anti-α-subunit RNA injected and the mean size of the AcCho-activated current in oocytes 4 days after injection. Bars (left to right) give measurements from the oocytes injected with *Torpedo* mRNA alone or *Torpedo* mRNA mixed with 10 ng, 1 ng, or 0.1 ng of anti-α-subunit RNA. Each column represents the mean ± SEM of five to seven measurements.

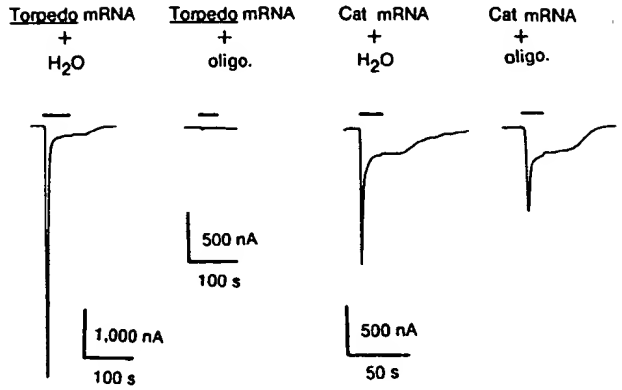

FIG. 5. Repression of AcChoR synthesis by an oligonucleotide. AcCho-activated currents in oocytes injected with *Torpedo* mRNA alone (≈50 ng), with *Torpedo* mRNA and oligonucleotide (≈150 ng), with cat mRNA (≈50 ng) alone, and with cat mRNA and oligonucleotide (≈150 ng). AcCho (100 μM) was applied by bath perfusion during the time indicated by bars. The oocytes were examined 3 days after injection.

DISCUSSION

We have shown previously that *Xenopus* oocytes injected with mRNA derived from *Torpedo* electric organ acquire functional AcChoRs and voltage-activated Cl^- channels on their surface membrane and that this depends on the translation of two different mRNAs (21). We now find that AcChoR subunit antisense RNAs block the expression of functional AcChoRs (Table 1) but not that of the Cl^- channels. This result alone indicates that the antisense RNAs specifically inhibit the expression of the AcChoR, a conclusion that is strengthened by the observation that receptors and channels expressed by other mRNAs were not inhibited by the *Torpedo* AcChoR subunit antisense RNAs. For example, when mRNA extracted from denervated cat muscle is injected into oocytes, it induces the appearance of muscle AcChoRs and voltage-activated Na^+ channels in the surface membrane (6, 26). This induction was not greatly affected by the injection of any AcChoR subunit antisense RNAs (unpublished results). Incidentally, this result suggests that the mRNAs coding for the *Torpedo* electric organ and cat muscle AcChoRs do not have sufficient homology to enable them to form very stable hybrid molecules between the *Torpedo* AcChoR antisense RNA and the cat muscle AcChoR mRNA.

It is known that an antisense RNA injected into *Xenopus* oocytes forms a hybrid molecule with the corresponding mRNA and prevents its translation (27, 28). Furthermore, it has been shown that the entire antisense RNA is not required to inhibit the translation of the mRNA, although it is most effective (see ref. 1). In the case of globin, a 45-base antisense RNA covering only the 5′ untranslated region of the mRNA and an antisense RNA that exposes only the 5′ untranslated region were as effective as the entire mRNA in blocking translation (27). In contrast, antisense RNAs that were complementary to the 3′ half of the protein coding sequence and/or 3′ untranslated sequence were unable to prevent translation. Therefore, it was suggested that the 5′ region of the mRNA must be covered by the antisense RNA to prevent translation effectively (27). A similar conclusion was reached with the thymidine kinase and chloramphenicol antisense RNAs (7). Our results (Fig. 3*B*) again suggest that an important antisense region for repressing the translation of the AcChoR's α-subunit mRNA is the 5′ region of the mRNA, but we show further that it is not necessary to cover the ribosome binding site or the initiation codon. For instance, the antisense RNA from the *Pvu* II-digested α-subunit cDNA, which does not cover the ribosome binding

site nor the AUG codon, was still quite effective in inhibiting the expression of functional AcChoRs. Interestingly the oligonucleotide (19-mer) designed to hybridize to the coding sequence for amino acid residues 4–10 of the α subunit was practically as effective as the entire antisense RNA.

In contrast to the observations mentioned above, a 270-base antisense RNA complementary to only the 3′ end of the mRNA coding for ribosomal protein L1 repressed translation as effectively as the entire (1300 bases) antisense RNA (28), whereas a 140-base antisense RNA that covered the 5′ untranslated region and the initiation codon was much less effective. These results appear to indicate that the accessibility of the target mRNA to the antisense RNA and its ability to form a stable hybrid molecule are critical factors for repressing translation, regardless of the region of complementarity.

Although all of the four subunit antisense RNAs that we used were effective in repressing translation, there was always some residual AcChoR activity, even when a large excess of antisense RNA was injected. Furthermore, the responses to AcCho appeared to increase with longer incubation times. This was most obvious in the case of the oocytes injected with the δ-subunit antisense RNA. At present we are unable to explain the basis for these observations. However, some residual activity might result if AcChoR molecules consisting of only three subunits are able to form functional receptors by themselves or by replacing the missing subunit with another subunit. In this context it should be noted that oocytes injected with combinations of three subunit-specific mRNAs that included the α-subunit mRNA have been shown to respond to AcCho, although the currents elicited were much smaller than those obtained with the complete AcChoR molecule (29). The largest response (about 10% of that of the complete AcChoR) was obtained from oocytes injected with the combination of α-, β-, and γ-subunit antisense mRNAs. This is consistent with our findings that the δ-subunit antisense RNA was the least effective in preventing the appearance of the functional AcChoR in the oocyte's surface membrane.

One of our objectives was to see how general is the inhibition of mRNA translation with antisense RNAs and to see if this could be applied to the study of oligomeric neurotransmitter receptors. We have demonstrated that the functional expression of the multisubunit AcChoR can be inhibited with any one of the subunit-specific antisense RNAs and that this inhibition was fairly stable for at least 1 week. Furthermore, we have shown that it is not necessary to use a full-length antisense RNA for efficient inhibition and that even a small synthetic oligonucleotide is effective in preventing translation.

Another important objective was to test the possibility of using antisense RNA for screening a cDNA library. A most efficient way of screening is to use hybridization probes such as oligonucleotides and antibodies. However, this requires the prior purification of the desired protein, which in the case of some neurotransmitter receptors is very difficult. Since *Xenopus* oocytes are very sensitive detectors of specific mRNAs coding for neurotransmitter receptors and voltage-operated channels (6, 17, 26, 30, 31), they can be used to screen a cDNA library for the genes encoding their structure. For that purpose, a library can be constructed by using a vector containing the SP6 or T7 promoter, or both, to allow the synthesis of sense or antisense RNA by *in vitro* transcription. The sense RNAs can be directly injected (cf. refs. 9 and 29) into the oocytes to test their ability to express the desired receptor, or the antisense RNAs can be coinjected with the whole mRNA, containing the desired receptor mRNA, into the oocytes to examine their ability to inhibit the expression of the receptor. When the expression of a functional receptor requires the synthesis of more than one type of protein subunit, an approach with antisense RNA may be of advantage, since we have shown that any one of the subunit antisense RNAs will inhibit the expression of functional receptors. Furthermore, as we have shown, full-length cDNA clones are not a necessary requirement for inhibition by antisense RNA. In contrast, when using sense RNA, full-length cDNA clones must be obtained, and all subunit mRNAs may need to be injected into the oocyte for expression of functional activity. All this might hinder the use of sense mRNA screening in the cloning of some heterooligomeric receptors or membrane ionic channels.

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Inhibition of Ly-6A Antigen Expression Prevents T Cell Activation

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Summary

Antisense oligonucleotides complementary to the 5' end of the mRNA encoding the Ly-6A protein were used to block the expression of that protein. Using this approach we could inhibit the expression of Ly-6A by 60–80% in antigen-primed lymph node (LN) T cells as well as in the D10 T cell clone. Inhibition of Ly-6 expression resulted in the inability to restimulate in vitro, antigen-primed T cells. It also blocked the activation of normal spleen cells by Con A, monoclonal antibody (mAb) to CD3, and mAb to Ly-6. In contrast, stimulation of normal spleen cells with the pharmacological agents PMA + ionomycin were unaffected by the inhibition of Ly-6 expression. Similar results were obtained with the D10 T cell clone; stimulation with Con A + interleukin 1 (IL-1), antigen-presenting cells (APC), or the clonotypic antibody + IL-1 was greatly reduced in the presence of antisense oligonucleotides to Ly-6. Stimulation with PMA + ionomycin was again unaffected. We also studied the effect of antisense oligonucleotides on stimulation of preactivated D10 cells. Preactivation of D10 cells with Con A + IL-1 renders them receptive to secondary stimulation by other lymphokines. In this case, antisense oligonucleotides to Ly-6 had no effect on secondary activation with IL-2, IL-4 + IL-1, or PMA + ionomycin. We conclude from these studies that Ly-6 expression is required for T cell receptor (TCR)-mediated T cell activation.

The murine Ly-6 alloantigens are encoded by members of a multigene family located on chromosome 15 (1–3). The Ly-6 alloantigens are small phosphatidylinositol (PI)[1]-anchored, membrane glycoproteins with a molecular weight of 15,000–18,000 (4–6). Members of the Ly-6 family are expressed on the surface of a large number of different cell types, including T cells, B cells, thymocytes, macrophages, neutrophils, and BM cells (7, 8). Recent studies have shown that the expression of the Ly-6A-encoded protein is associated with $CD4^+$ T cell activation and the acquisition of immunocompetence during T cell maturation in the thymus (9–13). It has been postulated that Ly-6 antigens, specifically Ly-6A, are involved in the transduction of signals originating in the CD3-TCR in $CD4^+$ cells. We addressed this question by specifically inhibiting Ly-6A antigen expression using antisense oligonucleotides, and here we show that inhibition of Ly-6A antigen expression resulted in a dramatic inhibition of antigen- and mitogen-driven T cell activation without affecting lymphokine or pharmacologically induced T cell response. Thus, it appears as though the Ly-6A antigen plays an important role in physiological T cell activation through the CD3/TCR complex.

[1] *Abbreviations used in this paper*: FGG, fowl gamma globulin; PI, phosphatidylinositol; PPD, purified protein derivative of tuberculin.

Materials and Methods

Oligonucleotides. Antisense (5'-AGTGTGAGAAGTGTCCAT-3') and two control oligonucleotides (5'-GGTCAACGGTGAGGC-CAT-3', 5'-TCACACTCTTCACAGGTA-3') were synthesized using a DNA synthesizer (model 380A; Applied Biosystems, Foster City, CA). Oligonucleotides were purified on OPC columns (Applied Biosystems) according to the standard procedure recommended by the company, or by polyacrylamide gel electrophoresis followed by elution and passage over a Sephadex G-50 column (Pharmacia Fine Chemicals, Piscataway, NJ). After purification, oligonucleotides were resuspended in PBS before use.

Mice. BALB/c and C3H/HeJ female mice were purchased from the Jackson Laboratories, Bar Harbor, Maine.

T Cell Proliferation Assay. Mice were primed with 100 μg of fowl gamma globulin (FGG; Cappel Laboratories, Malvern, PA) in CFA (Difco Laboratories, Detroit, MI) in the hind footpad. 8 d later, draining popliteal lymph nodes were removed and 3×10^5 lymph node cells were cultured in 100 μl of RPMI-1640 media

(Gibco Laboratories, Grand Island, NY), supplemented with antibiotics, L-glutamine, 1% fresh mouse serum, and 5×10^{-5} M 2-ME in a 96-well flat-bottomed tissue culture plate (Falcon Labware, Lincoln Park, NJ). At time 0, antisense oligonucleotides at a concentration of 7.0 μM in PBS or PBS alone were added to cell cultures. 5 or 50 μg of FGG or 50 μg of purified protein derivative of tuberculin (PPD; Staten Serum Institute, Copenhagen, Denmark) were added to cultures 0, 24, or 48 h after addition of oligonucleotides. Cultures were incubated for 72 h after the addition of antigen. Cell proliferation was assayed by the addition of 1.0 μCi [^{3}H]thymidine (6.7 Ci/mmol; New England Nuclear, Boston, MA) during the last 12–16 h of culture. Cells were then harvested and processed for liquid scintillation measurement of radioactivity.

Stimulation of the D10.G4.1 T Cell Clone. D10.G4.1 T cells (a conalbumin plus I-A^k-specific T cell clone) were added to culture at a concentration of 2×10^4 cells/well in 100 μl of RPMI-FCS media. Antisense oligonucleotides at a concentration of 7.0 μM in PBS or PBS alone were then added to these cell cultures. 24 h later, D10 cells were stimulated with either 10 ng of the anticlonotypic mAb 3D3 + 10^{-7} M rIL-1, 50 μg/ml conalbumin + 10^5 mitomycin C-treated AKR spleen cells (which serve as APC), 1.5 μg/ml Con A + 10^{-7} M rIL-1, or a mixture of 1.0 μg/ml of PMA and 10.0 μg/ml of ionomycin. Cultures were incubated for 72 h after the addition of antigen, antibody, or pharmacological agents. Cell proliferation was assayed as described above.

Lymphokine-induced Proliferation of Preactivated D10.G4.1 Cells. D10.G4.1 T cells were activated with 1.5 μg Con A + 10^{-7} M IL-1 for 72 h to make these cells receptive to IL-2- and IL-4-mediated proliferation (14). Activated D10 cells were then harvested, washed, and added to culture at a concentration of 2×10^4 cells/well in 100 μl of RPMI-FCS media. Antisense oligonucleotides at a concentration of 7.0 μM in PBS or PBS alone were then added to these cell cultures. 24 h later, D10 cells were stimulated with either 10 ng of mAb 3D3 + 10^{-7} M rIL-1, 100 U of rIL-2, 500 U of rIL-4 (Amgen Corp., Thousand Oaks, CA) + 10^{-7} M rIL-1, or a mixture of 1.0 μg/ml of PMA and 10.0 μg/ml of ionomycin. Cultures were incubated for 72 h after the addition of antigen, antibody, or pharmacological agents. Cell proliferation was assayed as described earlier.

In Vitro Stimulation of Naive Spleen Cells. Spleen cells from naive BALB/c mice were added to culture at a concentration of 2×10^5 cells/well in 100 μl of RPMI media containing 10% FCS. Antisense oligonucleotides at a concentration of 10.0 μM were added at time 0. 24 h later, cells were stimulated with either 1.5 μg/ml of Con A; 145.2C11, an anti-CD3 mAb (19); 70.94, an anti-Ly-6A mAb (1); or with a mixture of 1 μg/ml of PMA and 10 μg/ml of ionomycin. Cultures were incubated for 72 h after the addition of antigen, antibody, or pharmacological agents. Cell proliferation was assayed as described earlier.

Flow Cytometry. 10^6 viable cells were resuspended in 100 μl PBS + 0.1% sodium azide, and stained with biotin-labeled 70.94 (anti-Ly6A), biotin-labeled J1j (mAb anti-Thy-1), FITC-RL-172 (rat mAb anti-mouse CD4), RM2-2 (rat mAb anti-mouse CD2), and 2C11 (hamster mAb anti-mouse CD3). FITC-streptavidin (Zymed Laboratories, South San Francisco, CA), FITC-F(ab')$_2$ mouse anti-rat Ig (Jackson ImmunoResearch, West Grove, PA), or FITC-IgG fraction of goat anti-hamster Ig (Cappel Laboratories) were used as the second stage reagents. Fluorescence analysis was carried out on an EPICS Profile Analyzer (Coulter Immunology, Hialeah, FL).

Results and Discussion

It was recently shown that the presence in vitro of DNA oligonucleotides complementary to a portion of mRNA encoding a particular protein (antisense oligonucleotides) can effectively inhibit the translation of that protein in cultured cells (for review see reference 15). We first studied the effect of antisense oligonucleotides directed against the Ly-6A mRNA on secondary in vitro stimulation of in vivo primed LN cells. As depicted in Fig. 1 *A*, LN cells primed to FGG in CFA responded poorly to FGG and to PPD when restimulated in vitro in the presence of Ly-6A antisense oligonucleotides. Control cultures without oligonucleotides or with

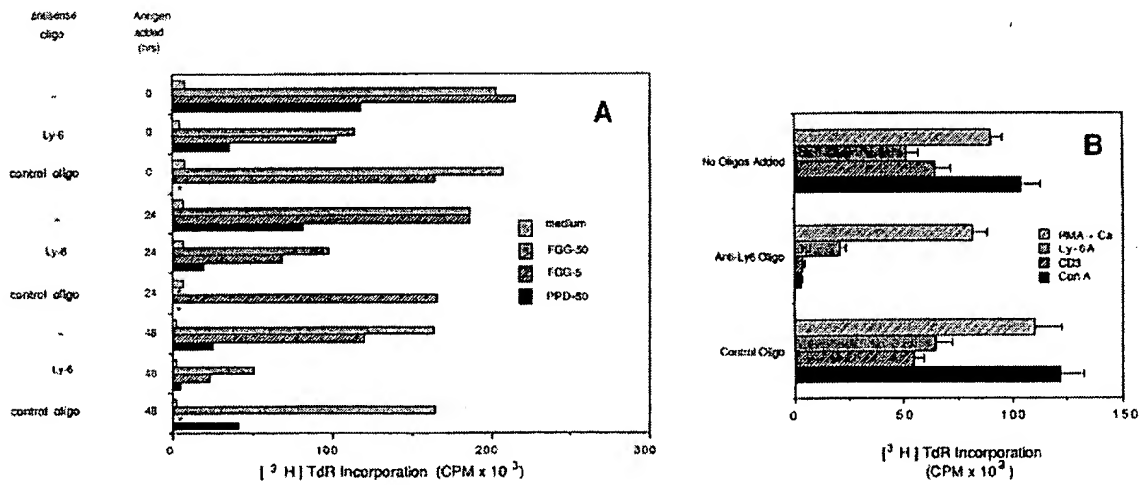

Figure 1. Inhibition of T cell proliferation by antisense oligonucleotides to the Ly-6A antigen. (*A*) FGG/CFA primed LN cells were restimulated in vitro with 5 or 50 μg/ml FGG or 50 μg/ml PPD 0, 24, or 48 h after the addition of oligonucleotides. Cell proliferation was assayed 72 h after the addition of antigen (Materials and Methods). All assays were performed in triplicate. Standard deviations were omitted for simplicity and were generally within 10–15% of the mean. (*) Not done. (*B*) Naive spleen cells were stimulated in vitro with PMA + ionomycin (PMA + Ca), mAb to Ly-6A (Ly-6A), mAb to CD3 (CD3), or Con A 24 h after the addition of oligonucleotides. Cell proliferation was assayed 72 h later. Results are presented as the mean response of triplicate cultures ± SEM.

irrelevant oligonucleotides responded very well. In this particular experiment, inhibition of proliferation was least apparent at time 0 and most apparent 48 h after the addition of antisense oligonucleotides. This was not always the case; in some experiments, the peak of inhibition was seen 24 h after the addition of the antisense oligonucleotides.

In Fig. 1 *B*, normal spleen cells were stimulated with the T cell mitogen Con A, anti-CD3 mAb, anti-Ly-6A mAb, or the combination of PMA and ionomycin, a calcium ionophore. In the presence of Ly-6A antisense oligonucleotides T cell activation was always inhibited except when the pharmacological agents PMA and ionomycin were used to induce cellular proliferation (Fig. 1 *B*). It is important to note that all the activation signals that were inhibited by Ly-6A antisense oligonucleotides have been previously shown to be delivered through the CD3/TCR complex (11, 14, 16).

We then tested whether Ly-6A antisense oligonucleotides could inhibit the proliferation of a cloned $CD4^+$, antigen-specific T cell line. We used the T cell clone D10.G4.1 (D10), a conalbumin plus I-A^k–specific Th2 T cell clone (17). D10 cells were stimulated with either antigen plus I-A^{k+} APCs, Con A + IL-1, 3D3 (an anticlonotypic mAb, 18) + IL-1, or PMA + ionomycin. The results illustrated in Fig. 2 show once again that activation with anti-TCR antibodies, mitogen, and antigen was inhibited in the presence of Ly-6A antisense oligonucleotides but not in the presence of an irrelevant oligonucleotide (Fig. 2). Again, activation by PMA + ionomycin in the presence of Ly-6A antisense oligonucleotides was unaffected.

The activation pathway of the D10 clone allowed us to further define the stage of activation where the Ly-6A antigen is required. It was previously shown that the D10 clone first requires an activation signal such as Con A + IL-1 in order to be receptive to stimulation by other lymphokines such as IL-2 or IL-4 + IL-1 (14). In the experiments shown in Fig. 3, D10 cells were first activated with Con A + IL-1 for 3 d, washed, and restimulated with IL-2, IL-4 + IL-1, 3D3 + IL-1, or PMA + ionomycin. In this case, the presence of Ly-6A antisense oligonucleotides had no effect on the secondary activation by IL-2, IL-4 + IL-1, or PMA + ionomycin. Only the physiological activation by the clonotypic antibody 3D3 + IL-1 was greatly reduced, since a preactivation step is not required. It seems therefore that once D10 cells receive the first activation signal by Con A + IL-1, Ly-6A is no longer required for activation by other lymphokines.

The inhibitory effects of Ly-6A antisense oligonucleotides on T cell activation appear to be due to the inhibition of Ly-6A expression during activation. Flow cytometric analysis of LN cells stimulated with FGG in the presence of Ly-6A antisense oligonucleotides showed that the expression of Ly-6A membrane antigens was specifically reduced by 60–80% as compared with cells stimulated in the absence of oligonucleotides or in the presence of irrelevant oligonucleotides (Fig. 4). The expression of Thy-1 (another PI-linked protein), CD2, CD3, and CD4, all of which were shown to be involved in T cell activation, was unaffected (Fig. 4). Similar selective reduction of Ly-6A antigen expression by Ly-6A antisense oligonucleotides was also seen on D10 cells stimulated by Con A + IL-1 (data not shown).

These results indicate that the expression of the Ly-6A protein on the cell surface is required for TCR-mediated activation of $CD4^+$ T cells. Thus, activation by antigen + MHC, mitogen, and anti-TCR antibodies was inhibited by Ly-6A-

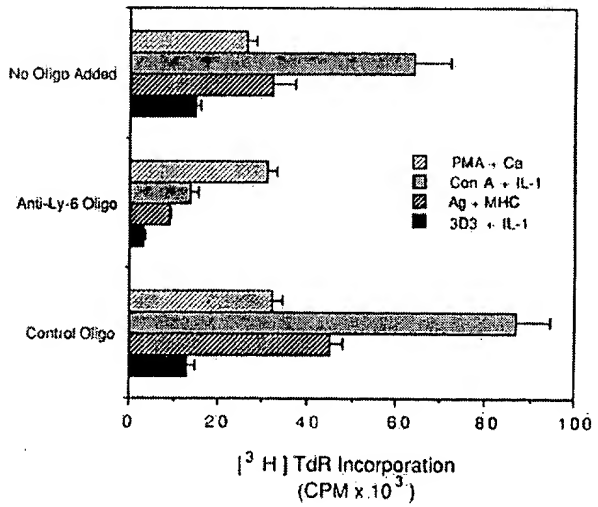

Figure 2. Inhibition of proliferation of the antigen-specific T cell clone D10.G4.1 by antisense oligonucleotides to the Ly-6A antigen. 2×10^4 D10 cells were cultured for 24 h with antisense or control oligonucleotides and then stimulated with PMA + ionomycin (PMA + Ca), Con A + IL-1, conalbumin + APCs (Ag + MHC), or the mAb 3D3 + IL-1. Cell proliferation was assayed 72 h later. Results are presented as the mean response of triplicate cultures ± SEM.

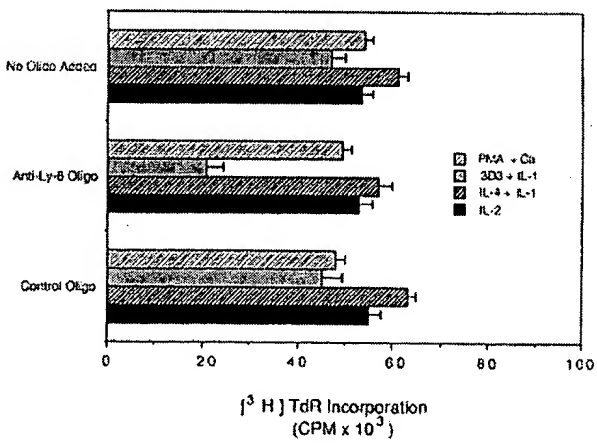

Figure 3. Antisense oligonucleotides to Ly-6A do not inhibit lymphokine-induced proliferation of preactivated D10.G4.1 cells. D10 cells were first activated with Con A + IL-1 for 72 h. 2×10^4 activated D10 cells were then cultured for 24 h with antisense or control oligonucleotides followed by stimulation with PMA + ionomycin (PMA + Ca), the mAb 3D3 + IL-1 (3D3 + IL-1), IL-4 + IL-1, or IL-2. Cell proliferation was assayed 72 h later. Results are presented as the mean response of triplicate cultures ± SEM.

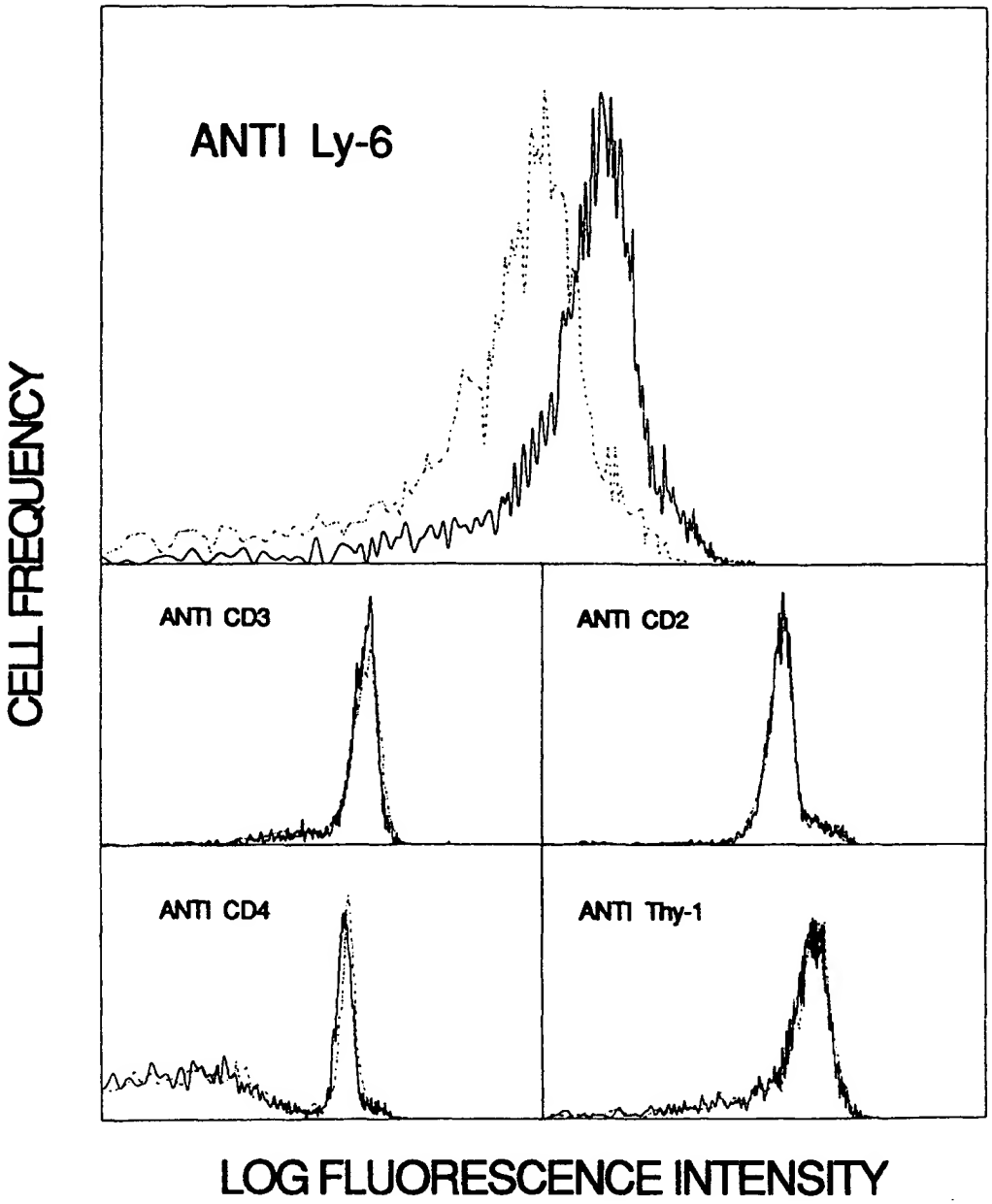

Figure 4. Inhibition of membrane Ly-6A antigen expression on cells treated with antisense oligonucleotides to Ly-6A. Primed LN T cells (J11d + complement-treated LN cells) were cultured for 24 h with antisense or control oligonucleotides and then stimulated with 50 μg/ml of antigen. 24 h later cells were harvested and stained with anti-Ly-6A, anti-CD2, anti-CD3, anti-CD4, or anti-Thy-1. (*Broken lines*) cells from cultures containing Ly-6A antisense oligonucleotides. (*Solid lines*) cells from cultures containing control oligonucleotides.

specific antisense oligonucleotides, while lymphokine and pharmacological activation were unaffected. The fact that antisense oligonucleotides can inhibit APC-dependent activation (mitogen, antigen + MHC, and anti-Ly6A mAb [9, 19]) and APC-independent responses (anti-CD3 and anticlonotypic mAb + IL-1) precludes the possibility that the antisense oligonucleotides inhibit T cell activation by inhibiting the expression of Ly-6 antigens only on APC (20). Furthermore, flow cytometric analysis confirmed the fact that Ly-6A antisense oligonucleotides specifically inhibited Ly-6A expression on T cells and T cell clones and did not effect expression of CD2, CD3, and CD4, all of which are surface proteins important in T cell activation. It was previously shown that T cell hybridomas lacking the expression of Ly-6A, or cells treated with phosphatidylinositol phospholipase C (PI-PLC) to remove all PI-linked proteins (including all Ly-6 proteins),

show markedly decreased responses to activation signals transduced via the TCR (13). It was also shown that crosslinking of the Ly-6 surface proteins by mAbs resulted in T cell activation, which in the case of Ly-6A antigens, was dependent upon the presence of the CD3/TCR complex (11) since T cells that did not express the CD3-TCR complex were refractory to stimulation by crosslinking Ly-6A antigens (11, 12). These as well as other studies (9–13) strongly suggested that Ly-6-encoded proteins are involved in TCR-mediated signal transduction. Our studies indicate that Ly-6A-encoded proteins are an absolute requirement for optimal activation induced by this type of signal transduction.

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